Departamento de Bioquímica

Facultad de Medicina

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“Angiotensina II y Gremlin activan la vía Smad durante la transición epitelio-mesenquimal, un mecanismo básico de progresión de daño renal:”.

Gisselle Carvajal González

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TESIS DOCTORAL

Gisselle Carvajal González
Licenciada en Medicina
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Servicio de Nefrologia
Fundación Jiménez Díaz

Don Jesús Egido de los Ríos, Catedrático de Medicina, y Doña Marta Ruiz-Ortega, Profesora Contratada Doctor de la Universidad Autónoma de Madrid.

CERTIFICAN

Que Doña Gisselle Carvajal González, Licenciada en Medicina por la Universidad de Chile, ha realizado bajo su dirección el trabajo titulado "Angiotensina II y Gremlin activan la vía Smad durante la transición epitelio-mesenquimal, un mecanismo básico de progresión de daño renal", que presenta como Tesis Doctoral para alcanzar el grado de Doctor por la Universidad Autónoma de Madrid.

Y para que conste, firmamos la presente en Madrid, 21 de Abril de 2010.

Dr. Jesús Egido de los Ríos

Dra. Marta Ruiz-Ortega

Dr. José González Castaño

Profesor Dto. Bioquimica.

Vivir no es sólo existir, sino existir y crear,

saber gozar y sufrir y no dormir sin soñar.

Descansar, es empezar a morir.

Gregorio Marañón

A mis padres

Bueno, hay tanto que agradecer que es difícil saber por dónde empezar …. Así que creo que lo mejor es partir por el principio de esta historia.

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Resumen

La transición epitelio-mesenquimal (TEM) es un proceso que contribuye de forma importante a la fibrosis renal. Entre los factores implicados en la progresión del daño renal, destaca Angiotensina II (AngII) como un elemento clave en la fibrosis. De hecho, los fármacos que bloquean AngII son una de las mejores estrategias terapéuticas actuales en patología renal. El objetivo de esta tesis ha sido investigar los mecanismos moleculares y mediadores implicados en la TEM. Para ello se han realizado estudios *in vitro*, *in vivo* y en biopsias de pacientes, utilizando técnicas de biología molecular y bioquímica. Los resultados demuestran que AngII induce TEM en el riñón a través de la activación directa, independiente de TGF-β, de la vía Smad. La regulación de este proceso es compleja e implica la activación de otros sistemas de señalización intracelular (como MAPK y RhoA/ROCK), y la síntesis endógena de mediadores profibróticos, como TGF-β y CTGF. En conjunto, estos datos sugieren que el bloqueo de la activación de Smad o de la síntesis endógena de CTGF son dos potenciales dianas terapéuticas en fibrosis renal. Además, explican los efectos órgano-protectores del bloqueo de AngII. El estudio de biopsias de pacientes con diversas glomerulopatías progresivas mostró sobre-expresión de Gremlin en áreas de fibrosis túbulo-intersticial, co-localizado con TGF-β, Smad activado y marcadores de TEM. Factores claves en el daño renal, como AngII y TGF-β, regulan la expresión renal de Gremlin. En estudios *in vitro*, Gremlin induce cambios típicos de TEM, a través de la activación de la ruta TGF-β/Smad. Estos datos sugieren que Gremlin puede ser considerado como un nuevo mediador profibrótico, y amplían los conocimientos sobre los procesos moleculares implicados en la fibrosis renal y sugieren nuevas dianas terapéuticas para las enfermedades renales crónicas.

Summary

Epithelial-mesenchymal transition (EMT) is an important process that contributes to renal fibrosis. Among the factors involved in the progression of renal damage, Angiotensin II (AngII) highlights as inducer of fibrosis. Drugs that block AngII actions are one of the best treatments to slow the progression of kidney diseases. The aim of this thesis was to investigate the molecular mechanisms and mediators involved in EMT. For this purpose, different approaches were done: *in vitro, in vivo* and in biopsies studies, using various techniques of molecular biology and biochemistry. The results presented in this study show that AngII induces EMT in the kidney through direct activation of the Smad pathway, independently of TGF-β. The regulation of this process is complex and involves the activation of other intracellular signaling systems (such as MAPK and RhoA/ROCK), and endogenous synthesis of profibrotic mediators such as TGF-β and CTGF. Taken together, these data show that inhibition of Smad pathway and endogenous synthesis of CTGF could be good therapeutic strategies against renal fibrosis. In addition, these findings explain the renoprotective effect of drugs that block AngII by acting at the cellular level. The study of biopsies from patients with several progressive glomerulopathies showed over-expression of Gremlin in areas of tubulointerstitial fibrosis in co-localization with TGF-β, Smad activation and EMT markers. Gremlin is regulated in the kidney by key factors in renal damage, such as AngII and TGF-β. Our *in vitro* studies demonstrated that Gremlin induces typical changes of EMT through activation of TGF-β/Smad pathway. These data suggest that Gremlin can be considered as a new mediator of fibrosis. These results extend the knowledge about the molecular processes involved in renal fibrosis and suggest new therapeutic targets for chronic kidney disease.

CLAVE DE ABREVIATURAS

Relación de abreviaturas que aparecen en el texto. En muchos casos se ha conservado la correspondiente abreviatura en inglés debido a su frecuente utilización en el lenguaje científico.

ALK	Quinasa similar a la activina
AMP	Aminopeptidasas
Ang	Angiotensina
AP-1	Proteína activada 1
BMP	Proteína morfogenética del hueso
CTGF	Factor de crecimiento del tejido conectivo
ECA	Enzima convertidora de Angiotensina
ERC	Enfermedad renal crónica
Fsp-1	Proteína específica de fibroblasto
IL	Interleuquina
LAP	Péptido asociado a la latencia
LTBP	Proteína de unión al TGF-β latente
MAPK	Proteínas quinasas activadas por mitógeno
MCP-1	Proteína quimioatractante de monocitos 1
MEC	Matriz extracelular
MMPs	Metaloproteinasas
NF-κB	Factor Nuclear κB
NO	Oxido nítrico
OPN	Ostopontina
R-Smads	Smads reguladas por receptor
SMA	Actina de músculo liso
SRAA	Sistema Renina Angiotensina Aldosterona
PAI-1	Inhibidor de Activador del Plasminógeno 1
PDGF	Factor de crecimiento derivado de plaquetas
PKC	Proteínas quinasa C
RANTES	Regulated on Activation, Normal T Expressed and Secreted
ROS	Especies reactivas de oxígeno
TEM	Transición epitelio-mesenquimal
TGF-β	Factor transformador del crecimiento beta
TNF-α	Factor de necrosis tumoral α
TRI	Receptor de TGF-β tipo I

TRII	Receptor de TGF-β tipo II
TSP-1	Trombospondina 1
UUO	Obstrucción ureteral unilateral

I. INTRODUCCIÓN

1. La enfermedad renal crónica y sus mecanismos de progresión

Uno de los mayores desafíos de la nefrología actual es el número creciente de pacientes que progresan hasta insuficiencia renal terminal y requieren tratamiento de sustitución mediante diálisis o trasplante, lo que conlleva un deterioro en la calidad de vida de los pacientes y un alto coste para los sistemas de salud. La enfermedad renal crónica ha tenido un aumento explosivo en los últimos tiempos, con un crecimiento aproximado de un 7% anual, convirtiéndose en un grave problema de salud pública.[23] Esta patología, no sólo involucra el daño a nivel renal, sino también un aumento del riesgo cardiovascular, lo que favorece la elevada morbi-mortalidad de estos pacientes.[36,72,145] En la práctica clínica, la mayoría de las nefropatías progresan lentamente hacia la pérdida definitiva de la función. Independiente de la etiología, la progresión de la enfermedad renal se caracteriza por un proceso inflamatorio inicial, seguido de fibrosis túbulo-intersticial, atrofia tubular y glomeruloesclerosis.[120]

La fibrosis túbulo-intersticial juega un rol clave en la progresión del daño y es así como la severidad de los cambios túbulo-intersticiales se correlacionan mejor con la pérdida de función renal que con el grado de glomeruloesclerosis, lo que no es sorprendente ya que el túbulo-intersticio ocupa más del 90% del volumen renal. Basados en numerosas evidencias experimentales, los factores más importantes de daño túbulo-intersticial son la presencia y magnitud de la proteinuria y la activación del sistema renina angiotensina aldosterona (SRAA).[89] La proteinuria persistente influye en la progresión de las enfermedades glomerulares determinando una activación tubular de factores de transcripción como NF-κB y AP-1, que participan en la transcripción de genes proinflamatorios (MCP-1, RANTES, osteopontina) y genes profibrogénicos (TGF-β, PDGF), dando lugar a una reacción inflamatoria y a la fibrosis intersticial.[93] El SRAA tiene un papel fundamental en los cambios fisiopatológicos que llevan al fallo renal terminal. La investigaciones realizadas en los últimos años han mostrado que este sistema es, como se comentará posteriormente, muy complejo y su principal efector, la angiotensina II (AngII) ha pasado de ser considerado como un agente vasoactivo a una verdadera citoquina pro-inflamatoria y pro-fibrótica.[168]

2. Fibrosis renal

La respuesta normal tras un daño en el tejido renal es la reparación, con la intención de lograr la restauración completa de la arquitectura y función del tejido. Sin embargo, por razones que aún se desconocen, la regeneración completa de un tejido, es decir una

reparación sin cicatriz, ocurre únicamente en el tejido fetal, en el adulto sólo se logran grados variables de restauración del tejido, lo que lleva a la acumulación de tejido fibroso. [47,88]

La fibrosis consiste en la acumulación de matriz extracelular (MEC) en respuesta a una inflamación persistente. La síntesis de MEC, cuyo principal componente es el colágeno, es parte del proceso normal de reparación, pero su síntesis excesiva es deletérea ya que exacerba el daño creando un círculo vicioso de perpetuación.[22] Una vez sintetizado, el colágeno es remodelado mediante un proceso de proteólisis. La degradación del colágeno es llevada a cabo principalmente por las MMPs, una familia de endopeptidasas dependientes de Zinc. En condiciones patológicas, el desequilibrio entre la síntesis y la degradación de MEC lleva a la pérdida de función del tejido al ser reemplazado por tejido fibroso.[47,170]

La principal célula encargada de síntesis de MEC es el fibroblasto intersticial, el cual cuando está activo pasa a llamarse miofibroblasto y se reconoce por la síntesis *de novo* del marcador mesenquimático α-SMA. El aumento en el número de miofibroblastos intersticiales se correlaciona con la progresión de la enfermedad renal.[45,46] El miofibroblasto tiene diferentes orígenes ***(FIGURA 1)***, migración desde la región perivascular,[164] proliferación local de precursores residentes,[45-47] reclutamiento de precursores sanguíneos[39] y transformación a partir de células epiteliales o endoteliales mediante el proceso de transición epitelio-mesenquimal (TEM)[77] o transición endotelio-mesenquimal.[174]

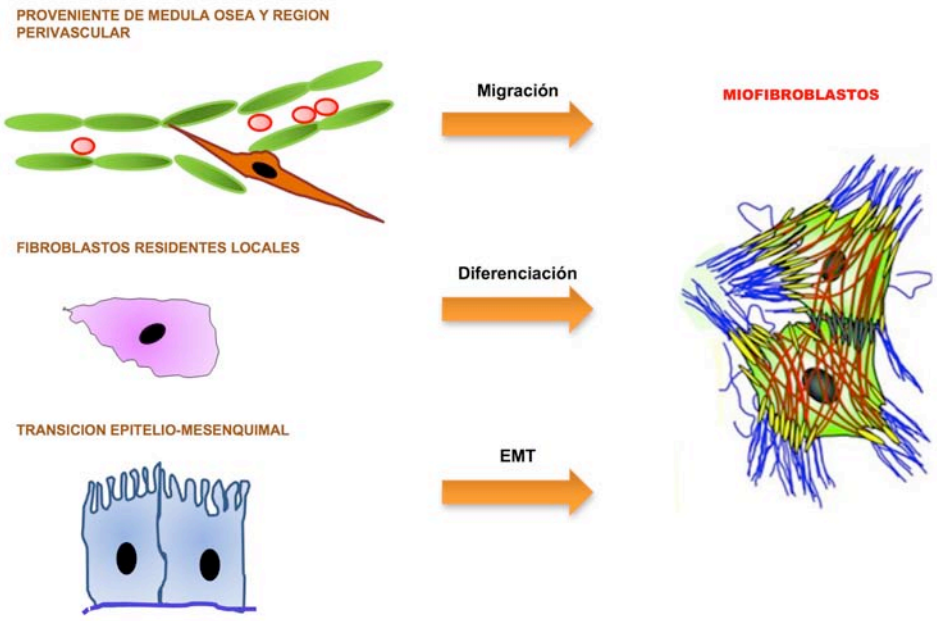

FIGURA 1: Origen de los miofibroblastos en el riñón dañado.

A pesar de la gran cantidad de estudios acerca de la patogénesis de la fibrosis renal, aún estamos lejos de conseguir estrategias terapéuticas para revertir el daño renal ya establecido. En los últimos años la TEM ha surgido como una importante vía de generación de miofibroblastos productores de MEC en la enfermedad renal, por lo que podría ser considerada como una potencial diana terapéutica.

3. Transición epitelio-mesenquimal (TEM)

La TEM es un proceso mediante el cual células epiteliales completamente diferenciadas adquieren un fenotipo mesenquimal. La mayoría de los túbulos del riñón adulto, con excepción de los colectores, provienen del mesénquima metanéfrico a través de un proceso llamado transición mesenquimo-epitelial, por lo tanto, se podría afirmar que en esencia la TEM sería un proceso de embriogénesis reversa, lo que demuestra una conexión entre la fisiología del desarrollo embrionario y la des-diferenciación celular de muchos estados patológicos.[67,152]

Las células túbulo-epiteliales y los miofibroblastos intersticiales presentan grandes diferencias en cuanto a morfología y fenotipo, además de encontrarse en compartimientos tisulares diferentes. En condiciones normales las células túbulo-epitelilales se encuentran unidas firmemente mediante varios mecanismos de adhesión. La E-cadherina es una proteína presente en las uniones adherentes que se une a los filamentos de actina intracelulares mediante cateninas y juega un rol esencial en mantener la integridad estructural del epitelio y su polarización. Uno de los eventos iniciales en el proceso de TEM es la pérdida en la expresión de E-cadherina, lo que lleva a la desestabilización estructural del epitelio, haciendo que las células se separen unas de otras y pierdan su polaridad.[172] En numerosos estudios se describe que en la TEM también hay disminución de la proteína ZO-1, componente fundamental de las uniones estrechas entre las células epiteliales.[175] Posteriormente se produce la reorganización del citoesqueleto y un aumento en la expresión de α-SMA, lo que no sólo define el cambio estructural de la célula, sino que también le confiere la capacidad contráctil que le permite la migración e invasión a otros compartimientos tisulares.[172] Además de los cambios en el citoesqueleto de actina, se observan otros cambios en la expresión de proteínas, como de citoqueratina (fenotipo epitelial) a vimentina (fenotipo mesenquimal). [112,154] Otro evento fundamental es la disrupción de la membrana basal tubular por la acción proteolítica de las MMP-2 y MMP-9 lo que permite la migración de las células transformadas hacia el intersticio.[77]

De todo lo anterior se desprenden cuatro eventos fundamentales en el proceso de TEM, que se muestra en la figura 2: ***(I)*** Pérdida de la adhesión epitelial; ***(II)*** expresión *de novo* de marcadores mesenquimales y reorganización de los filamentos de actina; ***(III)*** disrupción de la membrana basal tubular; ***(IV)*** la migración e invasión celular. [5,6,77,78,172]

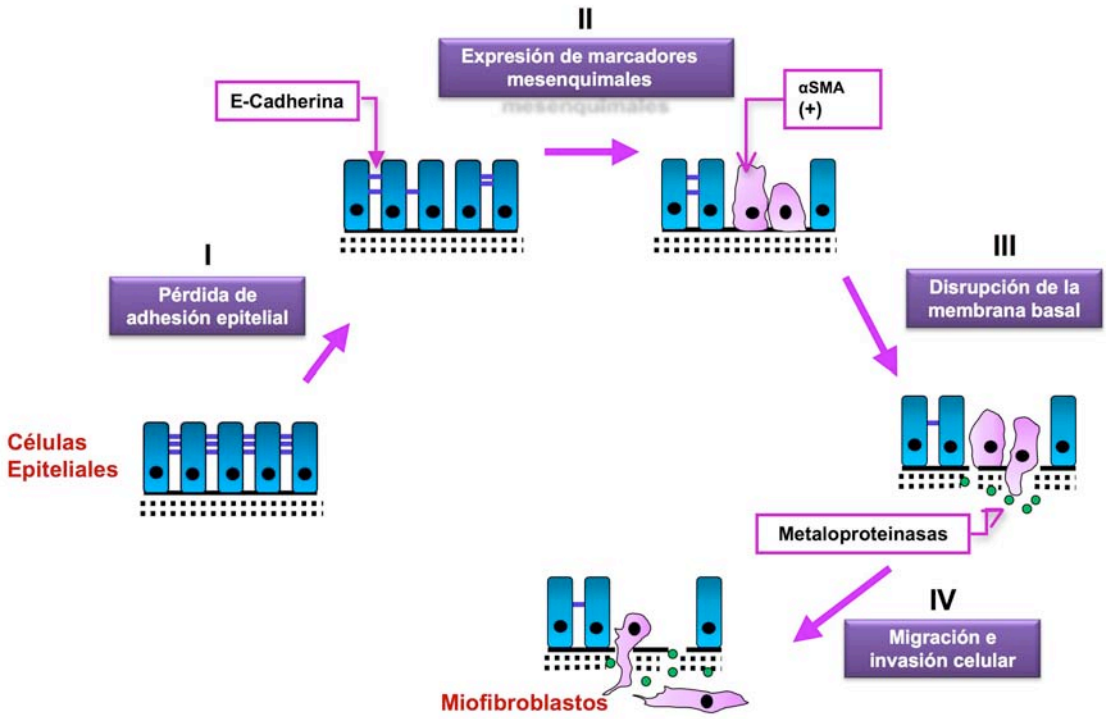

FIGURA 2: Etapas del proceso de transición epitelio-mesenquinal.

El primer estudio en demostrar la presencia de TEM en fibrosis renal, utilizó como marcador la proteína Fsp-1, que es específica fibroblastos y no se expresa en células epiteliales. En este trabajo utilizaron un modelo de enfermedad anti-membrana basal en ratón, donde se detectó expresión de Fsp-1 en células del epitelio tubular.[153] Estudios posteriores en varios modelos animales de daño renal han confirmado la existencia de TEM. Así, en un modelo de nefrectomía 5/6 se observó expresión *de novo* de α-SMA y de filamentos de actina en el epitelio tubular a las tres semanas de la nefrectomía, y posteriormente se evidenciaron cambios en la morfología celular y migración al intersticio.[106] En un modelo de obstrucción ureteral unilateral se ha descrito abundante co-expresión de α-SMA y del marcador epitelial E-cadherina, ambas características de células metaestables.[172] Un estudio posterior demostró que la TEM contribuye en alrededor de un 36% a la generación de miofibroblastos.[57]

La TEM es un proceso que involucra producción de citoquinas, factores de crecimiento, moléculas de adhesión y cambios en la composición de la matriz extracelular.[5,6,172] Por otra parte, existen diversos factores del microambiente peritubular y capilar que sirven de estímulo para el proceso de TEM. Esta lista está en constante crecimiento e incluye varias citoquinas como IL-1β y Oncostatina M, factores de crecimiento como TGF-β, EGF, VEGF y CTGF, proteasas como la MMP2 y factores de estrés ambiental como hipoxia/especies reactivas de oxígeno y productos de glicosilación avanzada. [67, 78,98,104, 123,152]

Las señales intracelulares, activadas por estos factores, implican la activación de complejos sistemas de señalización, como Ras/Rho GTPasas, quinasa de Rho (ROCK), tirosina quinasa Src, quinasa de unión a integrina (ILK), Wnt-1, Smad2 y Smad3, las quinasas de la cascada MAPKs, la fosfatidilinositol-3-quinasa, transactivación del receptor EGF y activación del factor nuclear NF-κB.[62,77,156] Además, existen rutas que se inhiben, como GSK-3β que da lugar a la estabilización y translocación nuclear de Snail, un potente represor transcripcional de E-cadherina y otras moléculas de adhesión e induce parada del ciclo celular y supervivencia.[7]

Cabe destacar que la TEM es un proceso reversible, al menos en las etapas iniciales. Además, hay moléculas que regulan de forma negativa la TEM y promueven la transición mesenquima-epitelial. Así, la proteína morfogenética del hueso 7 (BMP-7) ha demostrado bloquear y revertir la TEM, mediante la inducción del co-represor transcripcional Smad5, que interfiere con la activación de Smad2/3 inducida por TGF-β. Smad7 es otra molécula que regula de forma negativa la TEM, mediante el bloqueo de la fosforilación y activación de Smad2/3.[62,77,156]

4. El factor del crecimiento transformante beta (TGF-β)

El TGF-β pertenece a una superfamilia de factores de crecimiento formada por más de 40 miembros entre los que se encuentran activinas, inhibinas, factores de diferenciación de crecimiento y BMPs. Todos los miembros de la familia comparten secuencia y dominios estructurales. Son reguladores multifuncionales de la división, diferenciación, migración, adhesión, organización y muerte celular; además promueven la producción de MEC, la homeostasis y la embriogénesis.[59,85,97] Entre todas estas destaca el TGF-β con un papel crucial en la homeostasis tisular y en muchas patologías, incluyendo cáncer, enfermedades autoinmunes y cardiovasculares.

Se han descrito tres isoformas diferentes de TGF-β: TGF-β1, TGF-β2 y TGF-β3. TGF-β1 es la más importante para el sistema cardiovascular y renal. La síntesis de TGF-β es un proceso complejo ***(FIGURA 3).*** Diversos factores regulan la expresión génica de TGF-β, entre los que destaca AngII, estrés mecánico y altas concentraciones de glucosa.[134] Inicialmente TGF-β se sintetiza como una proteína inactiva llamada TGF-β latente. Consiste en una región principal y un péptido asociado a la latencia (LAP). Este péptido interacciona con proteínas de unión al TGF-β latente (LTBP) anclándolo a la MEC. El TGF-β se activa por digestión proteolítica, proceso en el que están implicados diversos factores como, trombospondina 1, plasmina, microambientes ácidos, MMPs y β6 integrina.[1,70] Se han descrito dos importantes receptores de TGF-β: el receptor TGF-β tipo I (TRI), también conocido como quinasa similar a la activina (ALK), y el receptor de TGF-β tipo II (TRII). Ambos son receptores transmembrana con actividad serina-treonina quinasa. El TGF-β se une al TRII induciendo un cambio conformacional, que le permite dimerizar con TRI e inducir su fosforilación, formando un complejo activo que transmite la señalización al interior de la célula.[59,86,97,124]

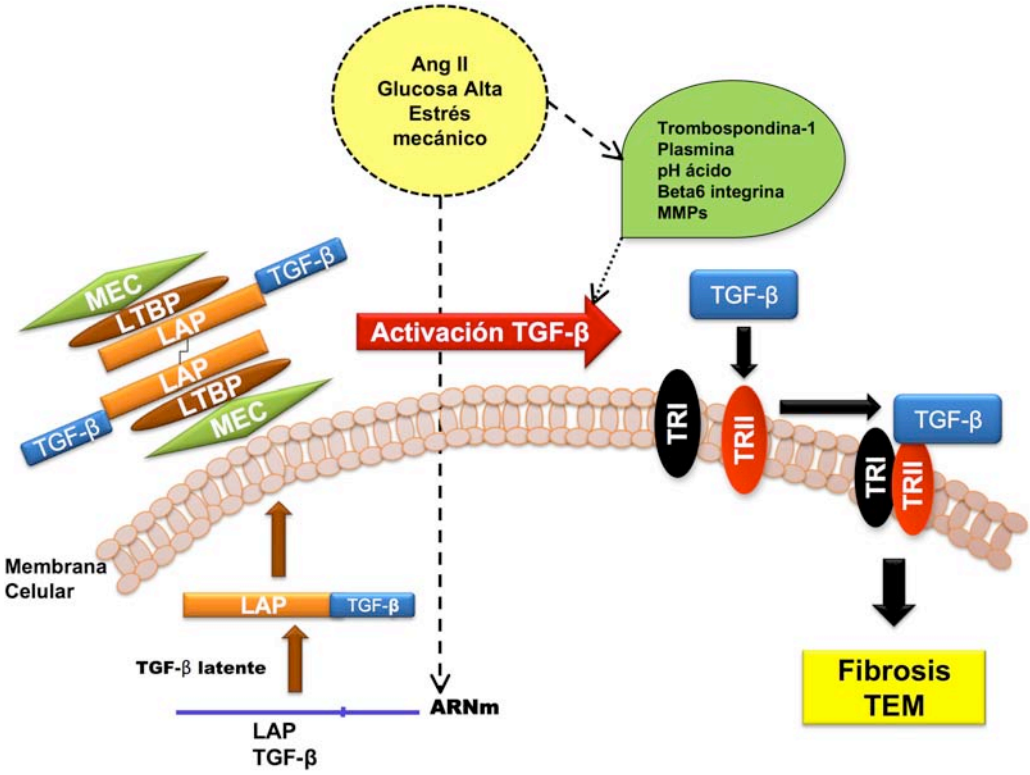

FIGURA 3: Síntesis de TGF-β y respuestas celulares.

La principal vía de señalización de TGF-β son las proteínas Smad ***(FIGURA 4)***. El primer evento en esta ruta es la fosforilación de las proteínas Smads reguladas por receptor (R-Smads), Smad2 y Smad3 en la serina C-terminal. Las R-Smads fosforiladas forman un complejo heterotrimérico con Smad4. Estos complejos se traslocan la núcleo y funcionan como

reguladores transcripcionales de genes diana. La unidad inhibitoria Smad7 ejerce su acción de dos formas: se une al TRI activo e impide la fosforilación de las R-Smads, o produce degradación proteosomal del complejo, reclutando a las ubiquitina ligasas Smurf1 y Smurf2.[134]

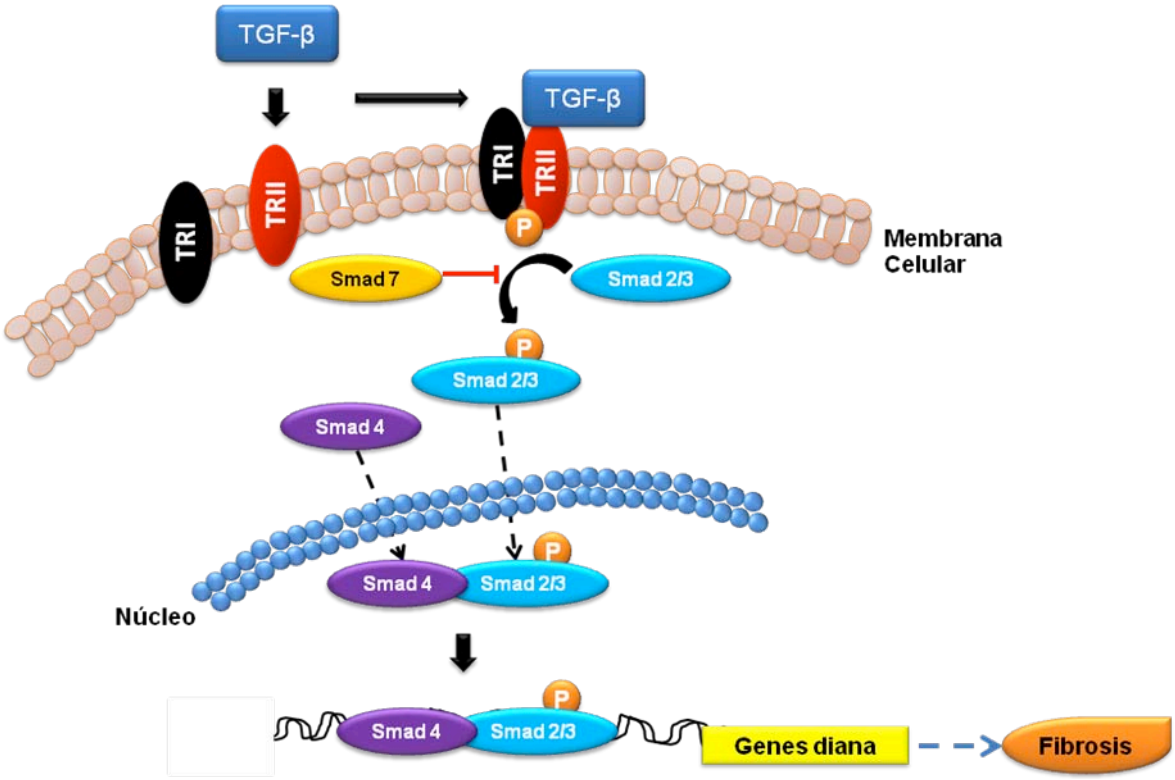

FIGURA 4: Vía de señalización Smad.

4.1 TGF-β y daño renal

TGF-β interviene en múltiples procesos fisiopatológicos a nivel renal, es capaz de estimular directamente la transcripción de un gran número de genes de matriz extracelular, inhibir la producción de colagenasas y estimular la expresión tisular del inhibidor de las metaloproteinasas, favoreciendo la acumulación de MEC. Además, TGF-β es capaz de inicar y completar todo el proceso de TEM. [33,67,172] Por estos motivos se le ha considerado como la principal citoquina profibrogénica en el riñón.[13,12,146]

La mayor parte de los estudios realizados en TEM se han centrado en la respuesta de TGF-β y sus mecanismos de señalización. Entre ellos destaca la ruta Smad,[123] la activación de proteínas quinasas, como la cascada MAPKs y la activación de la proteína G pequeña Rho y de su diana efectora ROCK, que son también mecanismos claves en la TEM.[4, 8,30,116.]

5. El sistema renina angiotensina aldosterona (SRAA)

Tradicionalmente el SRAA se ha considerado un sistema endocrino, en el cual a partir del Angiotensinógeno, producido en el hígado y mediante la acción de la Renina liberada en el aparato yuxtaglomerular renal, se genera el péptido inactivo AngI. Mediante la acción de la enzima convertidora de angiotensina (ECA) pulmonar se genera AngII. La AngII se une a receptores específicos en la corteza adrenal generando la liberación de Aldosterona. En esta visión clásica ***(FIGURA 5)***, la función principal del SRAA circulante es mantener la presión arterial mediante la vasoconstricción inducida por AngII y la retención de sodio a nivel del túbulo colector mediada por Aldosterona.[167]

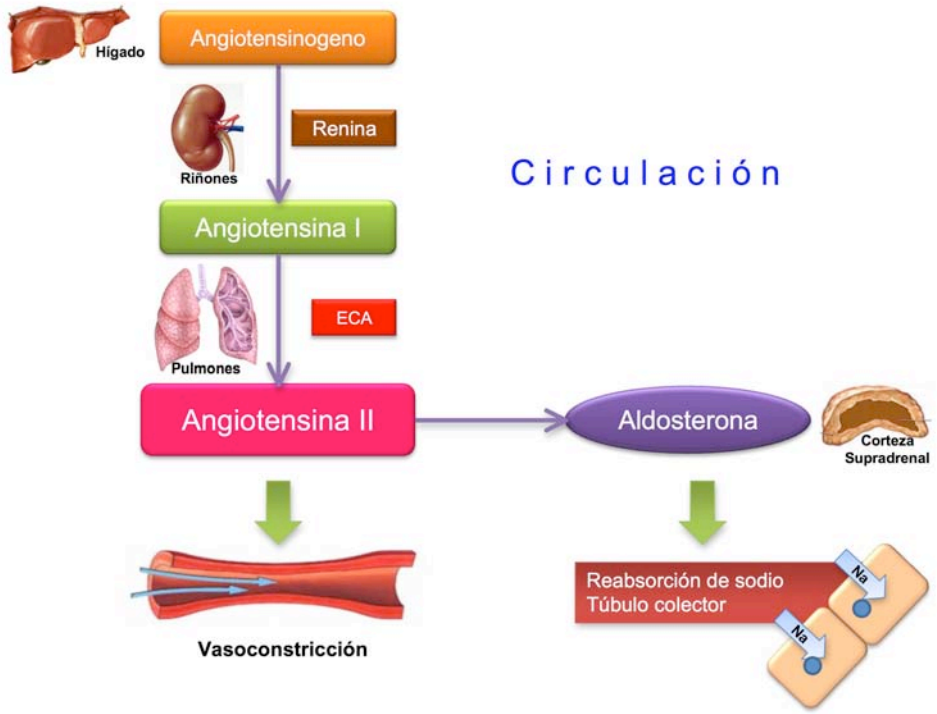

FIGURA 5: Visión clásica del sistema renina angiotensina aldosterona.

Además, se ha descrito un SRAA local que actúa en forma independiente del sistémico.[117] El SRAA circulante regula las respuestas fisiológicas, mientras que la activación del SRAA local contribuye a los procesos patológicos como proliferación celular, apoptosis, acumulación de MEC e inflamación ***(FIGURA 6).*** En múltiples patologías crónicas, tales como infarto al miocardio, hipertensión arterial y enfermedad renal crónica, se han descrito niveles tisulares elevados de componentes del SRAA.[133,155] Además los fármacos que bloquean el SRAA, como los inhibidores de la ECA y antagonistas de los receptores AT_1 han demostrado mejorar la evolución clínica de pacientes con enfermedades cardiovasculares y renales.[3,16,128]

Factores que inducen daño renal como hiperglicemia y proteinuria son capaces de activar el SRAA local a nivel renal.[103] En pacientes con diversas nefropatías crónicas, incluida la nefropatía diabética, se ha demostrado niveles elevados de ECA y aumento de producción de AngII en el riñón.[90] Existe otra enzima capaz de generar AngII a nivel local, la serina proteasa quimasa, la cual mediaría el 80% de la formación de AngII a nivel cardiaco y el 60% a nivel de vasos. Se ha observado sobre-expresión de quimasa en los túbulos de biopsias renales de pacientes con nefropatía diabética.[51]

En los últimos años es SRAA se ha vuelto más complejo ***(FIGURA 6)*** y se han descubierto nuevos componentes del sistema: ***1)*** ECA2, ***2)*** el receptor de Ang-(1-7) denominado Mas, ***3)*** el receptor de AngIV y ***4)*** el receptor de renina. La ECA2 es una enzima similar a la ECA que se expresa predominantemente en las células endoteliales vasculares, incluyendo las renales. Esta enzima también actúa sobre AngI generando Ang-(1-9), la cual potenciaría la vasoconstricción mediada por AngII. En un segundo paso la Ang-(1-9) mediante la acción de ECA o la endopeptidasa neutra (NEP) genera Ang-(1-7), la cual actúa a través de su receptor específico conocido como receptor Mas.[144]

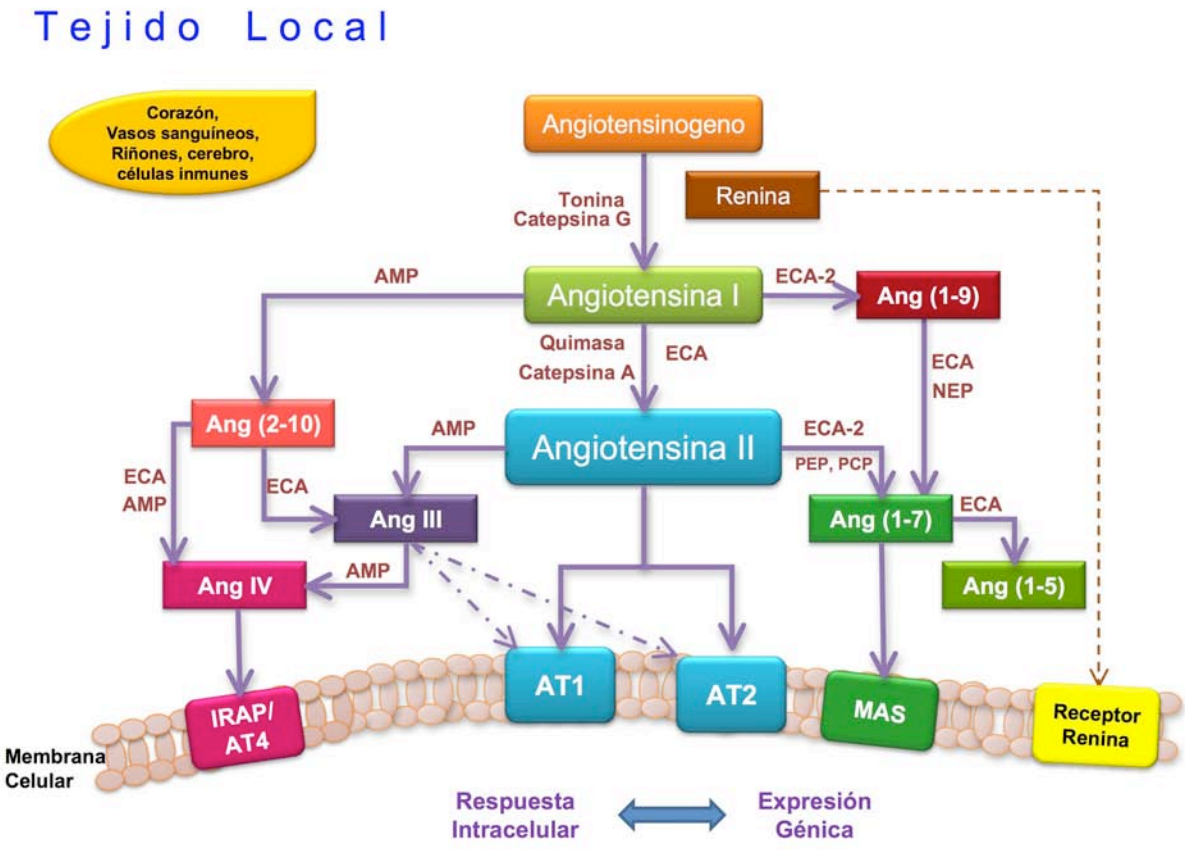

FIGURA 6. Visión actual del sistema renina angiotensina aldosterona.

La Ang-(1-7) tiene acciones contrarrestando parcialmente los efectos deletéreos de AngII, al menos en el sistema cardiovascular, donde se han realizado la mayoría de los estudios.[37,144] El rol de Ang-(1-7) a nivel renal está menos definido. Un estudio reciente mostró que la infusión de Ang-(1-7) induce inflamación vía activación del receptor Mas en el riñón.[31] Además la ausencia del receptor Mas retardó la progresión del daño renal en los modelos experimentales de obstrucción unilateral ureteral e isquemia/reperfusión.[31] En células renales se ha descrito que la Ang-(1-7) activa la ruta NF-κB e induce genes bajo su control, como MCP-1,[31] lo que sugiere un mecanismo potencial de estos efectos deletéreos renales.

Mediante la acción de aminopeptidasas (AMP), AngII es degradada en su porción N-terminal a AngIII, la cual comparte muchas de las acciones de AngII, señalizando a través de los mismos receptores e induciendo proliferación celular y producción de MEC a nivel renal. Las AMPs posteriormente degradan AngIII a AngIV.[2] En células vasculares la AngIV induce una respuesta inflamatoria, a través de la activación de NF-κB y podría contribuir a la fibrosis al regular PAI-1.[32] La AngIV se une a su receptor, denominado AT_4, que se expresa ampliamente en el riñón, a nivel endotelial y en los túbulos proximal y contorneado distal. Algunos autores afirman que la aminopeptidasa IRAP es el receptor AT_4, ya que une AngIV pero aún no se ha demostrado que esta unión sea la responsable de la activación de señales intracelulares.[130]

Recientemente se ha identificado el receptor de renina.[107,108] La activación de este receptor induce activación de MAPKs y la estimulación de moléculas profibróticas como PAI-1 y TGF-β.[52,53]

5.1 Angiotensina II y daño renal

La AngII, el principal péptido efector del SRAA, es actualmente considerada un factor de crecimiento implicado en la proliferación celular, acúmulo de MEC[28] y respuesta inflamatoria,[129,167] contribuyendo así a la patogenia de enfermedades crónicas como la hipertensión, la arteriosclerosis, la hipertrofia cardiaca y el daño renal.[131]

La AngII ejerce sus acciones biológicas mediante la unión a receptores específicos, ***(FIGURA 7)***, denominados AT_1 y AT_2.[26]

La mayoría de las acciones fisiológicas y fisiopatológicas de la AngII se realizan a través del receptor AT_1, tales como vasoconstricción, liberación de aldosterona y regulación de la matriz extracelular.[48,28,160] El rol del receptor AT_2 es menos claro ***(FIGURA 7)***. La activación del receptor AT_2 diminuye la presión arterial por liberación de óxido nítrico, además inhibe la

proliferación celular e induce diferenciación y apoptosis. Se ha demostrado que participa en el reclutamiento de células inflamatorias,[131,167] y en el daño tisular, donde se ha observado un aumento de su expresión.[131] En el sistema cardiovascular se ha descrito que los efectos del receptor AT_2 se opondrían a los del receptor AT_1, pero evidencias recientes indican que a nivel renal podría inducir activación de NF-κB, lo que apoyaría un efecto proinflamatorio.[166]

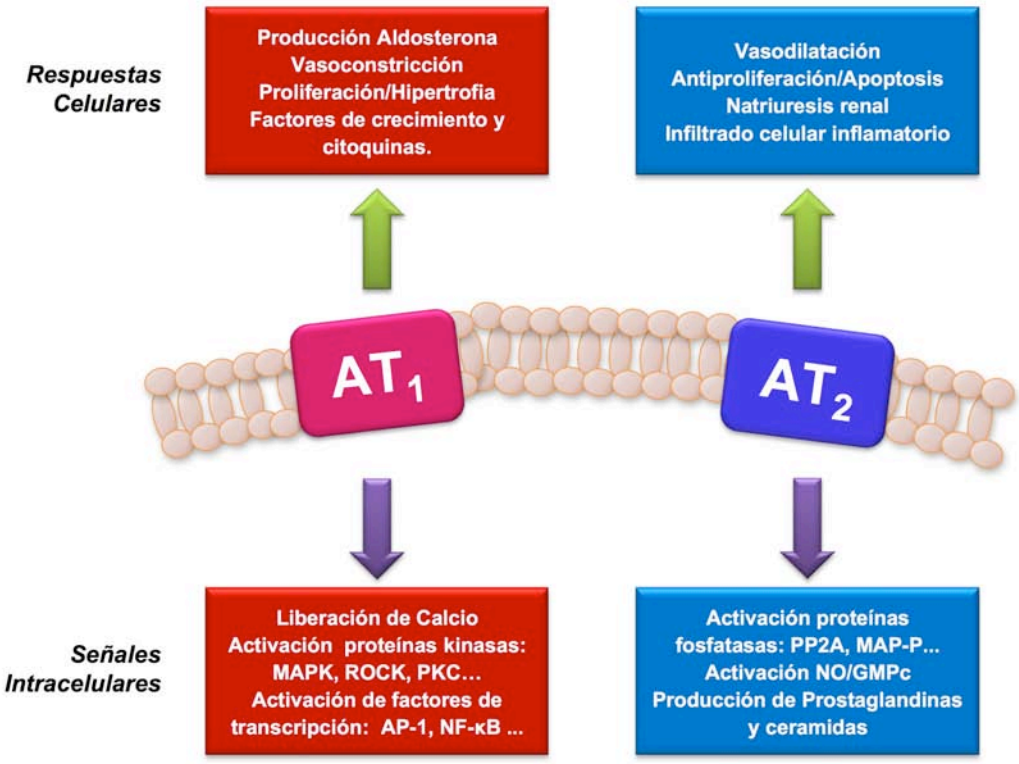

FIGURA 7. Respuestas celulares y mecanismos moleculares activados por AngII a través de sus receptores.

La AngII, vía receptores AT_1, activa proteínas G (Ras, Rho, Rac, entre otras), diversas proteínas quinasas (PKC, PTK, MAPK, Rho/ROCK), factores de transcripción (Jak/STAT, AP-1, NF-κB) y producción de radicales de oxígeno.[135,160] AngII induce la transactivación del receptor EGF, proceso que causa activación de señales intracelulares, como Ras, ERK, c-fos, Akt/proteína quinasa B y p70/S6 quinasa. Esta transactivación regula algunas respuestas de AngII, como el crecimiento celular y la expresión de fibronectina y TGF-β.[29] Las proteínas Rho participan en el daño cardiovascular y renal mediado por AngII.[140]

La AngII activa las células túbulo-epiteliales, fibroblastos intersticiales y células glomerulares, regulando el crecimiento celular, la síntesis de matriz extracelular y la producción de factores proinflamatorios.[90,136,169] Algunos de los efectos de la AngII están mediados por la producción endógena de diversos factores. Así, la hipertrofia/proliferación depende del equilibrio entre el PDGF y TGF-β, la producción de matriz extracelular está mediada por CTGF y TGF-β, [55,94,134,136,169,173] la adhesión de monocitos al endotelio vascular se produce vía VCAM-1 y el reclutamiento de células mononucleares está mediado por MCP-1. [132,136] ***(FIGURA 8).***

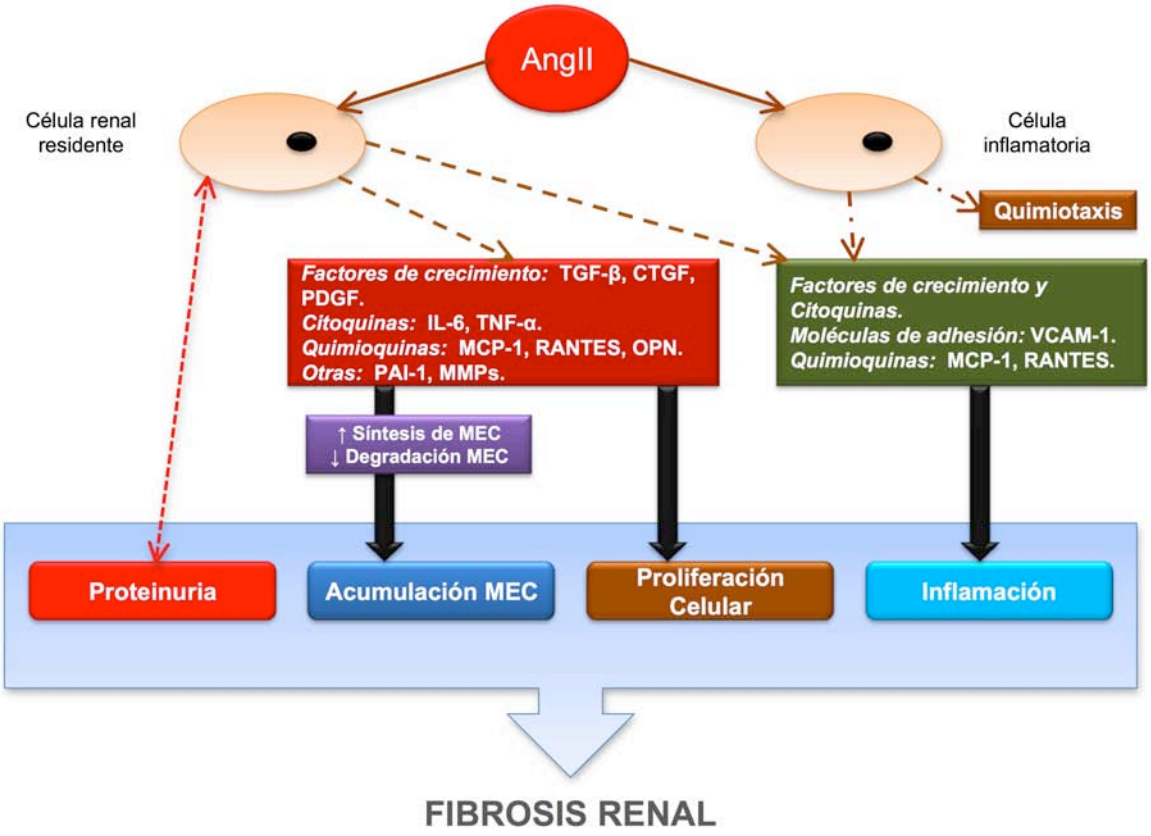

FIGURA 8. Mecanismos patogénicos de AngII en la enfermedad renal.

En el riñón existe una interesante relación entre AngII y TGF-β.[134] Se ha demostrado que los bloqueantes de AngII disminuyen la sobreproducción de TGF-β, la activación de la señales intracelulares y la fibrosis renal. [137,169] En células renales en cultivo, AngII estimula la expresión de TGF-β, y además el bloqueo de TGF-β modifica algunas respuestas de AngII, entre ellas la regulación de la MEC.[169] TGF-β y AngII comparten muchas respuestas celulares.[136,169] La mayoría de las acciones pro-fibróticas de TGF-β están mediadas por la

activación de la ruta de señalización de las proteínas Smad.[35,161] El bloqueo del receptor AT_1 disminuye la activación de la vía de señalización de las proteínas Smad en ratas con infarto de miocardio y en un modelo experimental de daño renal.[42,161]

Aunque TGF-β es uno de los principales factores que intervienen en la regulación de la fibrosis, las estrategias terapéuticas diseñadas para su bloqueo no han sido muy beneficiosas probablemente por sus propiedades anti-inflamatorias,[38] sin embargo, los fármacos inhibidores de AngII han demostrado ser una buena opción para bloquear TGF-β en humanos.[169]

Nuestro grupo ha sido el primero en demostrar que AngII activa la vía de señalización de las Smad, independiente de TGF-β, en células de músculo liso vascular,[125] por otra parte, la infusión de AngII a ratas induce daño tubular asociado a neo-expresión de α-SMA y Vimentina en células intersticiales.[15,60] Sin embargo, no existen estudios que investiguen si AngII es capaz de activar la vía Smad en riñón y su rol potencial en TEM.

6. El factor de crecimiento de tejido conectivo (CTGF)

El CTGF, también conocido como CCN2, es una proteína secretable, rica en cisteínas que fue identificada en el medio condicionado de células endoteliales de vena de cordón umbilical.[14] Esta proteína participa en procesos biológicos, como la regulación del ciclo celular, migración, adhesión y angiogénesis. En el riñón sano, CTGF no se expresa, pero este factor se induce en patologías renales humanas, incluyendo glomerulonefritis, glomeruloesclerosis y nefropatía diabética, correlacionándose sus niveles de expresión con la gravedad y la progresión de la fibrosis renal.[142] Su expresión está regulada por diversos factores implicados en el daño renal, entre los que destacan la AngII, TGF-β, altas concentraciones de glucosa y situaciones de estrés celular.

Se ha descrito que CTGF y TGF-β actúan de manera sinérgica para promover fibrosis crónica ***(FIGURA 9)***. En ratones, la coinyección subcutánea de ambos produce una fibrosis sostenida y persistente. En varios modelos experimentales, como la obstrucción unilateral del uréter, nefritis por anticuerpos anti-Thy1, nefropatía diabética, TGF-β y CTGF, se encuentran aumentados en etapas avanzadas de fibrosis, indicando que estos factores contribuyen a la progresión del daño renal.[142]

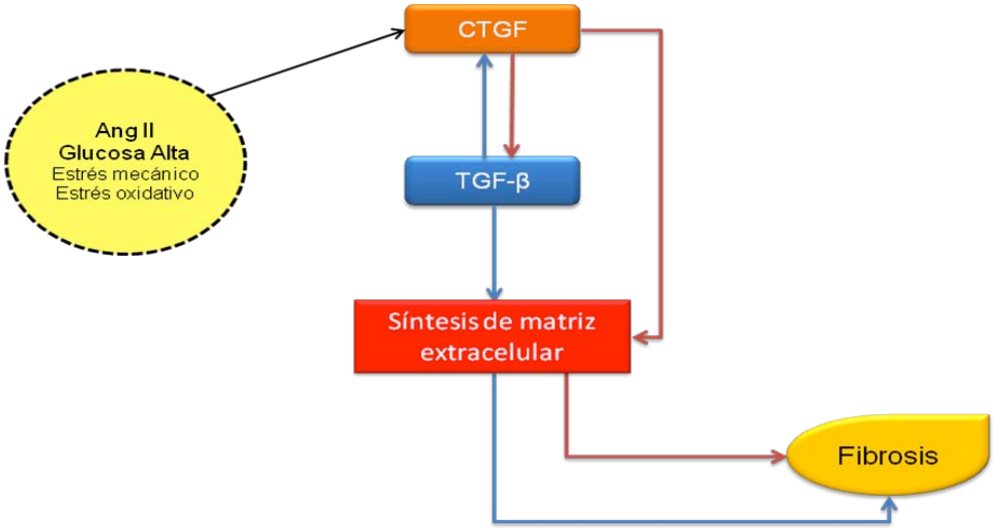

FIGURA 9. Regulación de las acciones de CTGF en fibrosis.

El tratamiento con antagonistas del receptor AT_1 e inhibidores de la ECA disminuyen la expresión renal de CTGF y la fibrosis en varios modelos experimentales de daño renal.[142] La infusión de AngII induce la expresión renal de CTGF rápidamente, apareciendo a los tres días, y precede en el tiempo a la acumulación de MEC (caracterizado por aumento en el depósito de fibronectina), observada tras una semana, indicando que CTGF puede actuar como mediador de la fibrosis renal causada por AngII *in vivo*.[139] Mediante estudios *in vitro* en células mesangiales de rata hemos observado que el bloqueo de la síntesis endógena de CTGF, mediante el uso de oligonucleótidos antisentido, previene la producción de fibronectina y colágeno IV causada por AngII.[139,143] CTGF promueve transdiferenciación de células tubuloepiteliales humanas a miofibroblastos *in vitro* y el bloqueo de CTGF da lugar a la inhibición de la transdiferenciación inducida por TGF-β[177] y por productos terminales de glicosilación avanzada.[17]

Nuestro grupo ha demostrado recientemente que la administración sistémica de CTGF en ratones causó una respuesta inflamatoria renal, caracterizada por reclutamiento de células inflamatorias al intersticio, activación del factor de transcripción NF-κB y producción de citoquinas proinflamatorias y factores quimiotácticos.[141]

Todos estos datos muestran que CTGF participa en el inicio y progresión del daño renal al ser capaz de inducir una respuesta inflamatoria y promover la fibrosis, señalándole como una posible diana terapéutica en el tratamiento de patologías renales.[142]

7. Gremlin

Gremlin es una proteína de 184 aminoácidos glicosilada, fosforilada y secretable.[159] Esta proteína contiene regiones ricas en cisteina y es un miembro de la familia DAN y se encuentra presente en la superficie celular, en el retículo endoplásmico y en el aparato de Golgi, presentando una forma soluble y otra asociada a células.

Gremlin actúa como antagonista de las BMPs, participando en procesos de crecimiento, diferenciación y desarrollo.[50] Ejerce su efecto a través de la unión directa y heterodimerización con BMP-2, 4 y 7, interfiriendo con la habilidad de estos ligandos de unirse a sus receptores. Por su característica de antagonista de BMPs jugaría un papel clave durante el proceso de nefrogénesis.[95] Muchos genes embrionarios que regulan la morfogénesis se vuelven silentes en el riñón adulto normal. Estudios recientes han demostrado la reactivación de genes del desarrollo en la patología renal, siendo Gremlin uno de ellos.[127]

Gremlin, también llamado Drm (down-regulated by mos), fue identificado como uno de los genes del desarrollo inducido en células mesangiales sometidas a altas concentraciones de glucosa por lo que inicialmente fue conocido como IHG-2 (induced in high glucose-2).[87] Estudios posteriores sugieren la participación de Gremlin como mediador de daño renal en la nefropatía diabética.[27,68,69,100] Así, un estudio reciente en un modelo de nefropatía diabética inducida por estreptozotocina en ratones heterocigotos para Gremlin mostró una disminución en la expresión de proteínas relacionadas con la fibrosis, como fibronectina y CTGF, en los ratones grem(+/-) comparado con los controles.[126] Se ha demostrado que Gremlin participa en otros procesos patogénicos como fibrosis pulmonar, fibrosis hepática y cáncer.[10,65,101,102] Sin embargo, no hay estudios evaluando las respuestas celulares directas de Gremlin sobre procesos de fibrosis.

Recientemente se han descrito una serie de funciones intracelulares de Gremlin, independientes de BMPs, las cuales mediarían respuestas celulares tales como la supresión de la tumorigénesis y la estimulación de la migración de células endoteliales.[19,20] Por lo tanto, Gremlin es una proteína con múltiples funciones, fisiológicas y patológicas, las cuales se realizan mediante mecanismos dependientes e independientes de BMPs.[148]

II. OBJETIVOS

OBJETIVOS

Objetivo General

Investigar los mediadores y los mecanismos intracelulares que participan en la transición epitelio-mesenquimal (TEM) en el riñón.

Objetivos Específicos

1. Evaluar si Angiotensina II (AngII) induce TEM en el riñón e investigar posibles mecanismos de señalización implicados en este proceso.

    1.1 Determinar si el factor transformador del crecimiento-β (TGF-β) es un mediador de la TEM inducida por AngII, evaluando el papel de la ruta TGF-β/Smad.

    1.2 Estudiar la participación de la activación de proteínas quinasas.

    1.3 Evaluar si el factor profibrótico CTGF regula esta respuesta de AngII.

2. Investigación de nuevos mediadores de fibrosis renal y TEM: Evaluación de Gremlin.

    2.1 Determinar la presencia de Gremlin en distintas nefropatías humanas. Estudiar si existe correlación con fibrosis renal y activación de la ruta TGF-β/Smad.

    2.2 Regulación de Gremlin por factores claves en la progresión del daño renal.

    2.3 Evaluar si Gremlin es capaz de inducir TEM *in vitro* y los mecanismos implicados.

III. MÉTODOS Y RESULTADOS

1. La Angiotensina II activa la ruta Smad durante el proceso de transición epitelio-mesenquimal en el riñón

La mayor parte de los estudios realizados hasta la fecha se han centrado en investigar los mecanismos implicados en la TEM causada por TGF-β. Así, se ha demostrado que la vía Smad y la activación de proteínas quinasas son claves en estos proceso.[4,8,30,116,123] Uno de los tratamientos con mejores resultados demostrados en clínica en patología renal es el empleo de bloqueantes de la AngII, ya que presentan efectos órgano protectores previniendo la progresión de la fibrosis renal.[3,16,128] Nuestra hipótesis de trabajo es que la AngII sería uno de los mayores factores profibróticos en el riñón, con una capacidad similar al TGFβ, en la inducción de fibrosis y TEM.

Los resultados de este trabajo demuestran que AngII es capaz de inducir TEM *in vivo*. Además, en células túbulo-epiteliales humanas en cultivo observamos que AngII induce TEM con una respuesta similar a la causada por TGF-β, confirmado que AngII es un importante factor profibrótico renal.

Uno de los objetivos de esta tesis ha sido estudiar si AngII es capaz de activar la ruta Smad en el riñón, y su posible implicación en la TEM, evaluando el papel de TGF-β en el proceso. Para ello, utilizamos un modelo experimental de infusión sistémica de AngII en ratas y realizamos estudios *in vitro* en células túbulo-epiteliales humanas, que se muestran en el siguiente trabajo.

Los resultados aquí presentados demuestran que a nivel renal la AngII activa la ruta Smad de forma directa e independiente de TGF-β, *in vivo* e *in vitro*, causando fibrosis renal. En los estudios realizados en células túbulo-epiteliales humanas en cultivo hemos observado que la activación de ruta AngII/Smads está regulada por la activación de las proteínas quinasas MAPKs, como se determinó por inhibidores farmacológicos. Utilizando un vector de expresión de Smad7, proteína que inhibe la activación de Smad2/3, demostramos que la TEM causada por AngII está mediada por la activación de las Smads. Además, la síntesis endógena de TGF-β está implicada en las respuestas de AngII observadas a tiempos largos, lo que sugiere que TGF-β participa en el mantenimiento de la fibrosis renal.

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Angiotensin II activates the Smad pathway during epithelial mesenchymal transdifferentiation

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Epithelial to mesenchymal transdifferentiation is a novel mechanism that promotes renal fibrosis and here we investigated whether known causes of renal fibrosis (angiotensin II and transforming growth factor β, TGFβ) act through this pathway. We infused angiotensin II into rats for 1 day and found that it activated the Smad pathway which persisted for up to 2 weeks in chronically infused rats. Renal TGF-β mRNA expression was increased at 3 days and its protein at 2 weeks suggesting Smad pathway activation occurred earlier than TGF-β upregulation. In cultured human tubuloepithelial cells, angiotensin II caused a rapid activation of Smad signaling independent of TGF-β however, Smad-dependent transcription after 1 day was TGF-β mediated. Two weeks of angiotensin II infusion activated genes associated with epithelial mesenchymal transdifferentiation. Stimulation with angiotensin II for 3 days caused transdifferentiation of the cultured epithelial cells by TGF-β-mediated processes; however, early changes were independent of endogenous TGF-β. Smad7 overexpression, which blocks Smad2/3 activation, diminished angiotensin II-induced epithelial mesenchymal transdifferentiation. Our results show that angiotensin II activates the Smad signaling system by TGF-β-independent processes, *in vivo* and *in vitro*, causing renal fibrosis.

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KEYWORDS: angiotensin; Smad; tubuloepithelial cells; fibrosis; TGF-β

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Activation of local renin–angiotensin system has been found in many kidney diseases. Angiotensin II (AngII), the main peptide of the renin–angiotensin system, contributes to the progression of renal damage.[1] This peptide activates tubuloepithelial cells, interstitial fibroblasts, and glomerular cells, regulating cell growth and extracellular matrix synthesis.[1–3] Many studies have demonstrated that AngII participates in renal fibrosis through endogenous production of growth factors.[1–4] The relation between AngII and transforming growth factor-β (TGF-β) in renal fibrosis is already known.[3] TGF-β transmits signals to the nuclei through the activation of the Smad pathway.[5,6] TGF-β binds to type II receptor, which activates the type I receptor kinase, which in turn phosphorylates the receptor-regulated Smads (R-Smads), Smad2 and Smad3, at C-terminal serines. The R-Smads then dissociate from the receptor complex to form a heterotrimeric complex with Smad4. These complexes translocate to the nucleus and function as transcriptional regulators of target genes. The inhibitory Smad7 binds to activated type I receptor, thereby preventing Smad2/3 phosphorylation, or recruits the ubiquitine ligases Smurf1 and Smurf2 to induce proteasomal degradation.[5,6] In renal cells, Smads participate in TGF-β-induced epithelial–mesenchymal transdifferentiation (EMT) and fibrosis.[6,7] The angiotensin type I receptor (AT_1) blockade diminishes Smad pathway activation in myocardial infarction in rats and in an experimental model of renal damage.[8,9] We have described that in vascular smooth muscle cells (VSMCs), AngII activates the Smad signaling pathway independently of TGF-β and linked to vascular fibrosis.[10] However, there are no studies investigating whether AngII activates the Smad signaling pathway in the kidney and its potential role in EMT.

Tubulointerstitial fibrosis is a final common pathway to end-stage chronic kidney diseases, and its severity correlates with renal prognosis. Many evidences suggest that renal tubuloepithelial cells can undergo EMT to become matrix-producing fibroblasts under pathologic conditions and, therefore, participate in the pathogenesis of chronic renal diseases. Cytokines, growth factors, adhesion molecules, and

changes in the extracellular matrix composition are involved in EMT.[11–13] TGF-β has been described as the most potent inducer of fibrosis and EMT.[5,7,11,14] In cultured tubulo-epithelial cells, AngII induces α-smooth muscle actin (α-SMA) expression and mesenchymal features.[15] Infusion of AngII into rats causes tubular injury associated with neoexpression of α-SMA and vimentin in renal interstitial cells;[16,17] therefore, it is reasonable to speculate that AngII could be involved in EMT in the kidney.

The aim of this work was to investigate whether AngII activates the Smad pathway in the kidney and its role in EMT, evaluating the involvement of endogenous TGF-β synthesis in these processes.

RESULTS

Systemic AngII infusion into rats activates the Smad pathway in the kidney

One of the initial steps of Smad activation is the phosphorylation of R-Smads.[5,6] We have investigated whether AngII *in vivo* could activate the Smad pathway in the kidney by evaluation of the time course evolution of R-Smad phosphorylation. In AngII-treated rats for 24 h total renal phosphorylated, Smad3 levels were significantly increased compared with control animals (western blot, Figure 1a), indicating a rapid Smad activation in the kidney. After 3 days, phosphorylated-Smad2/3 (p-Smad2/3) markedly appeared in the nucleus of tubuloepithelial cells (Figure 1b). Similar

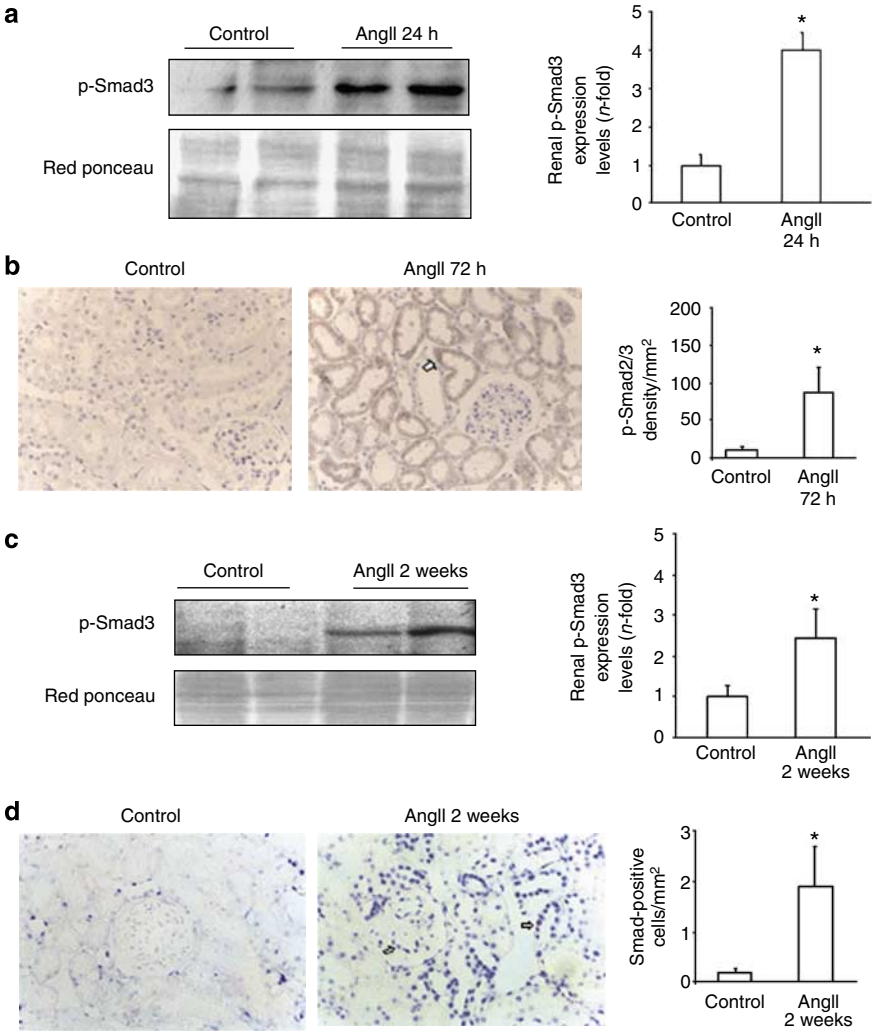

Figure 1 | AngII infusion activates the Smad pathway in the kidney. Rats were infused with AngII (100 ng/kg/min) from 24 h to 2 weeks and phosphorylated levels of Smad proteins were evaluated by (**a**, **c**) western blot and (**b**) immunohistochemistry. In western blot, phosphorylated-Smad3 (p-Smad3) levels were evaluated in renal protein extracts. Panels (**a**) and (**c**) show a representative experiment on the right panel and data as mean ± s.e.m. of 4–6 animals per group on the left. *$P<0.05$ vs control. Panel (**b**) shows a representative experiment of pSmad2/3 immunohistochemistry on the right and the quantification of data as mean ± s.e.m. of 4–6 animals per group on the left. (**d**) By Southwestern histochemistry, active Smad complexes, shown by nuclear blue staining (marked by arrows), were detected in AngII-infused rats for 2 weeks. The figure shows a representative experiment of three animals studied in each group on the right panel and data as mean ± s.e.m. of 4–6 animals per group on the left. *$P<0.05$ vs control. Original magnification × 200.

results were found with the antibody that recognizes only p-Smad3 (not shown). The Smad pathway remained activated in chronic AngII-infused rats, as shown by western blot (Figure 1c) and by Southwestern histochemistry, a technique that allows to detect Smad–DNA binding activity in paraffin-embedded tissues.[18] In AngII-infused rats for 2 weeks, active Smad complexes were observed in some glomerular and tubuloepithelial cells, whereas only a few positive cells were found in control animals (Figure 1d).

AngII-induced Smad activation occurs earlier than TGF-β production. To investigate whether Smad activation was directly induced by AngII or mediated by endogenous TGF-β synthesis, we have evaluated TGF-β mRNA and protein levels at different time points. AngII did not increase renal TGF-β mRNA levels after 24 h, but this gene was upregulated at 72 h and remained elevated after 2 weeks (real time PCR, Figure 2a). TGF-β is synthesized as an inactive protein, which is anchored to the cell membrane before activation.[6] In cultured cells, AngII increases TGF-β mRNA expression, protein production, and activation of latent TGF-β, the latter regulated by a thrombospondin-1 mediated process.[19] In rats that have been AngII-treated for 72 h, throsmbospondin-1 mRNA levels were not upregulated compared with controls (real-time PCR, not shown). Moreover, in these animals, there was no increase in TGF-β protein levels, which were dramatically overexpressed after 2 weeks of AngII infusion

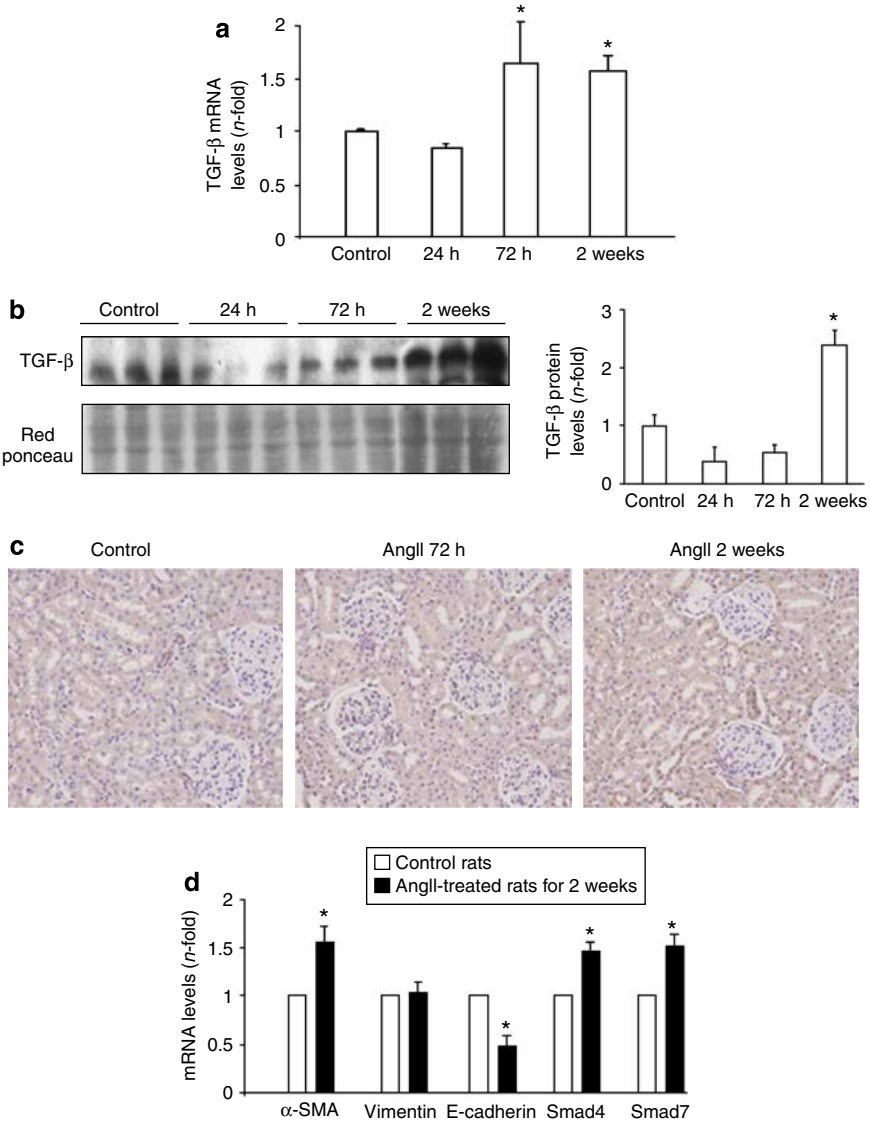

Figure 2 | Renal TGF-β expression is upregulated in chronic, but not acute, AngII infusion. Renal gene and protein expression levels of TGF-β were evaluated at different time points of AngII infusion. Panel (**a**) shows data of real-time PCR experiments as mean ± s.e.m. of 6–8 animals per group. *$P<0.05$ vs control. Panel (**b**) shows a representative experiment evaluated by western blot on the right panel and data as mean ± s.e.m. of 4–6 animals per group on the left. *$P<0.05$ vs control. Panel (**c**) shows a representative experiment of TGF-β immunohistochemistry. (**d**) Renal gene expression of EMT markers and Smad proteins in AngII-infused rats for 2 weeks. The figure shows data of real-time PCR experiments of Smad4, Smad7, E-cadherin, α-SMA, and vimentin gene expressions as mean ± s.e.m. of 6–8 animals per group. *$P<0.05$ vs control.

(Figure 2b and c). These data indicate that Smad activation (observed at 24 h) occurs before the increase in TGF-β synthesis (observed after 72 h), suggesting that AngII *in vivo* directly activates Smad pathway in the kidney.

Chronic infusion of AngII into rats induces EMT in the kidney. By means of real-time PCR, we have analyzed the renal expression of the mesechymal marker α-SMA at different time points of AngII infusion, and only after 2 weeks, α-SMA mRNA was upregulated. In these animals, vimentin was not modified and the epithelial marker E-cadherin was downregulated (Figure 2d). Renal gene expressions of Smad4 and Smad7 were also increased in AngII-infused rats compared with controls, indicating upregulation of Smads expression (Figure 2d). These data show that chronic infusion of AngII into rats causes renal fibrosis, associated with induction of EMT, overexpression of TGF-β, and activation of the Smad pathway.

AngII activates the Smad pathway in cultured human tubuloepithelial cells

To further demonstrate a direct effect of AngII in Smad pathway, *in vitro* studies were carried out in cultured human tubuloepithelial cells (HK2 cell line). In these cells, stimulation with AngII and TGF-β (1 ng/ml) induced a rapid phosphorylation of R-Smads at 15 min of incubation, demonstrated by a significant increase in nuclear levels of phosphorylated Smad2 and Smad3 (Figure 3a, western blot). By confocal microscopy, we have observed that, in growth-arrested HK2 cells, Smads proteins are located in the cytosol, as demonstrated using indirect immunofluorescency for Smad4 (Figure 3b). Treatment with AngII for 15 min increased total p-Smad2 staining and caused the nuclear translocation of p-Smad2 and Smad4 (Figure 3b).

In the nucleus, the R-Smad/Smad4 complex can activate transcription through direct binding to certain DNA sequences.[6] In tubuloepithelial cells, AngII increased DNA-binding activity to the CAGA box as early as at 10 min, with a maximal response at 15 min (3.5-fold, $P<0.05$ vs control, $n=3$ electrophoretic mobility shift assay experiments). The same was observed with TGF-β (Figure 4a). The specific Smad complexes were detected by competition assays with a 100-fold excess of unlabeled or mutant CAGA box (marked by arrows in Figure 4a). By supershift assays, we have observed that the antibodies against Smad2 and Smad4, alone or in combination, shifted the band to a higher molecular weight (Supershifted bands marked by arrows), supporting the involvement of Smad2 in AngII responses (Figure 4b).

We have investigated whether AngII regulates Smad-mediated gene expression by transient transfection with a luciferase Smad reporter plasmid (Smad/luc), which contains

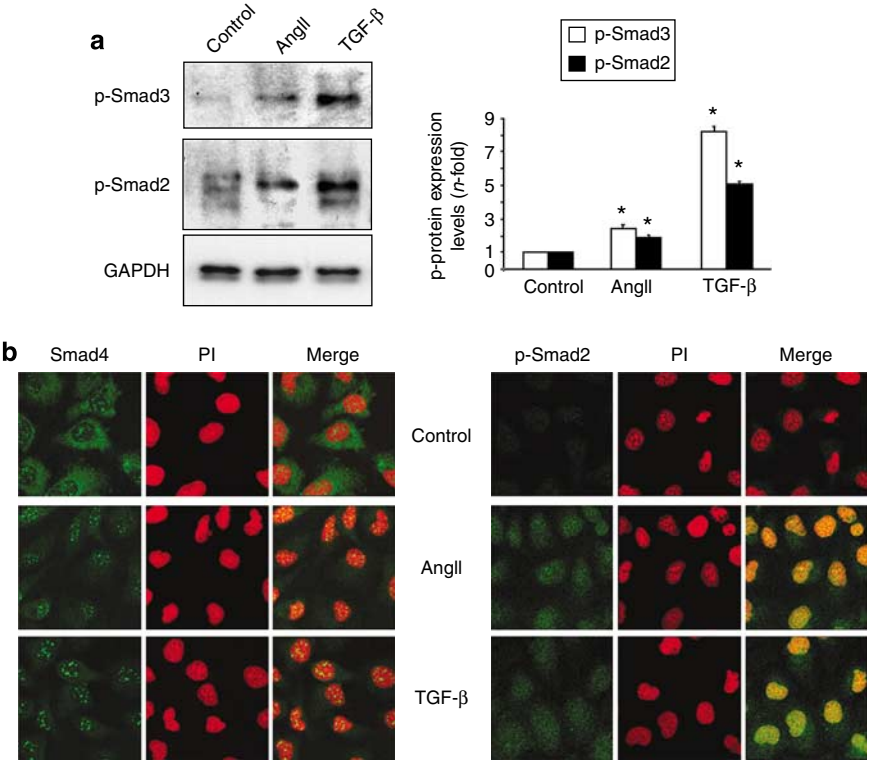

Figure 3 | AngII induces a rapid activation of Smad pathway in cultured human tubuloepithelial cells. Cells treated with 10^{-7} mol/l AngII or 1 ng/ml TGF-β for 15 min. (**a**) AngII induces Smad2/3 phosphorylation. Total protein levels of phosphorylated-Smad2 (p-Smad2) and p-Smad3 were quantified by western blot. The figure shows a representative experiment on the right panel and data as mean ± s.e.m. of three experiments on the left. *$P<0.05$ vs control. (**b**) AngII causes the nuclear translocation of Smad4 and p-Smad2. The evaluation of Smad4 and p-Smad2 was done by confocal microscopy with fluorescein isothiocyanate-labeled secondary antibodies (green staining). Nuclei were stained with propidium iodide (in red). In the merge of FITC and PI staining, the yellow staining indicates nuclear localization of Smads proteins. The results are representative of three independent experiments.

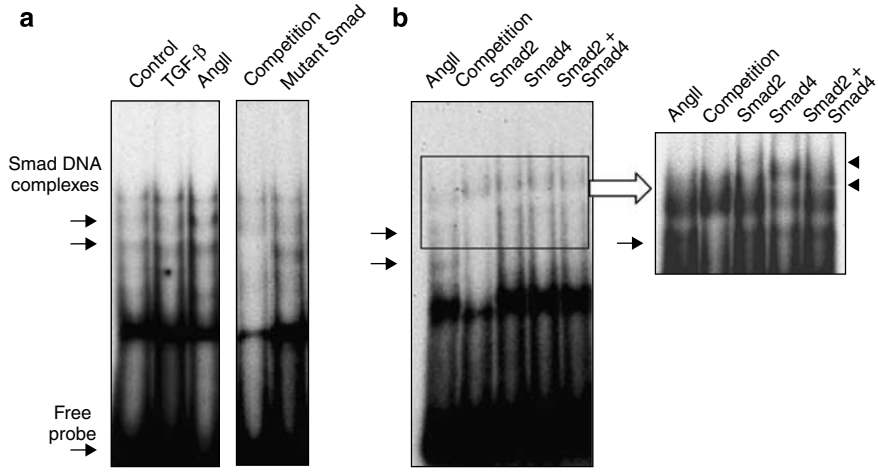

Figure 4 | AngII increases Smad DNA-binding activity in human tubuloepithelial cells. (**a**) HK2 cells were incubated with 10^{-7} mol/l AngII or 1 ng/ml TGF-β for 10 min. Smad activity was determined by electrophoretic mobility shift assay. Competition assays with a 100-fold excess of unlabeled or mutant CAGA box show specific Smad complexes (marked by arrows). (**b**) Identification of Smad complexes induced by AngII in HK2 cells. The nuclear extracts of AngII-treated cells were pre-incubated for 1 h with antibodies against the Smad2 and Smad4, alone or in combination, before incubation with labeled oligonucleotide. Protein complexes were resolved by electrophoresis (supershift). The enlarged picture corresponds to the area marked by upper arrow in (**b**). Supershifted bands are observed with anti-Smad2 and anti-Smad4 and with the combination of both antibodies (marked by arrowheads). Figure shows a representative electrophoretic mobility shift assay of three experiments.

four copies of the recognition site for the Smad sequence. After 24 h of incubation with AngII, the Smad promoter activity was increased (1.5-fold, $P<0.05$ vs control, $n=4$ experiments).

Mechanisms involved in AngII-induced Smad activation in cultured human tubuloepithelial cells

AngII rapidly activates Smad pathway by a TGF-β-independent mechanism. To block TGF-β, we used two different strategies: a neutralizing antibody against active TGF-β, which blocks AngII-induced extracellular matrix production,[4] and the SB431542 compound, an inhibitor of the activin receptor-like kinase 5.[20] In HK2 cells, the blockade of TGF-β did not modify AngII-induced Smad2/3 phosphorylation (Figure 5a) or their nuclear translocation (Figure 5b and not shown) observed after 15 min of incubation. Similar results were found in the regulation of Smad DNA-binding activity in cells treated with AngII for 15 min (not shown). These data show that early Smad activation by AngII is independent of endogenous TGF-β production or activation.

TGF-β mediates late activation of Smad pathway caused by AngII. The blockade of TGF-β diminished Smad-dependent transcription observed after 24 h of incubation with AngII (Figure 5c), suggesting that TGF-β is involved in long-term Smad activation.

Mitogen-activated protein kinase signaling mediates AngII-induced Smad activation. Previous studies in VSMCs have demonstrated a cross-talk between Smad and mitogen-activated protein kinase (MAPK) pathways,[10,21] but there are no studies in renal cells. The involvement of MAPKs cascade was evaluated using specific inhibitors of p38-MAPK (SB203580), extracellular signal-regulated kinase1/2 (ERK) (PD98059) and Jun N-terminal kinase (JNK) (SP600125) pathways.[22,23] We have found that in HK2 cells all three MAPK inhibitors (p38, ERK, and JNK) significantly diminished AngII-induced Smad2 phosphorylation (Figure 5d).

AngII causes EMT via AT_1 receptors in cultured human tubuloepithelial cells

Incubation with AngII for 3 days causes phenotypic conversion of HK2 cells. The transformed cells lost the typical cobblestone pattern of an epithelial monolayer and displayed a spindle-shaped, fibroblast-like morphology, assessed by phase contrast microscopy (Figure 6a). This effect was more marked after 5 days. AngII induced *de novo* protein expression of α-SMA and vimentin at 24 h and remaining elevated after 3 days. This was accompanied by the loss of the epithelial marker E-cadherin, essential for the structural integrity of renal epithelium (western blot; Figure 6b). In unstimulated tubuloepithelial cells, no staining for vimentin or α-SMA was observed by confocal microscopy. Treatment with AngII for 3 and 5 days induced vimentin and α-SMA-positive microfilaments in the cytoplasm, and E-cadherin immunostaining disappeared (Figure 6c). These data suggest that tubuloepithelial cells, stimulated with AngII, undergo a conversion process into myofibroblasts.

Pre-incubation of HK2 cells with the specific AT_1 antagonist valsartan blocked AngII-induced vimentin expression and changes in cell morphology (Figure 6d and e). These results suggest that AngII-induced EMT was mediated by AT_1 receptors.

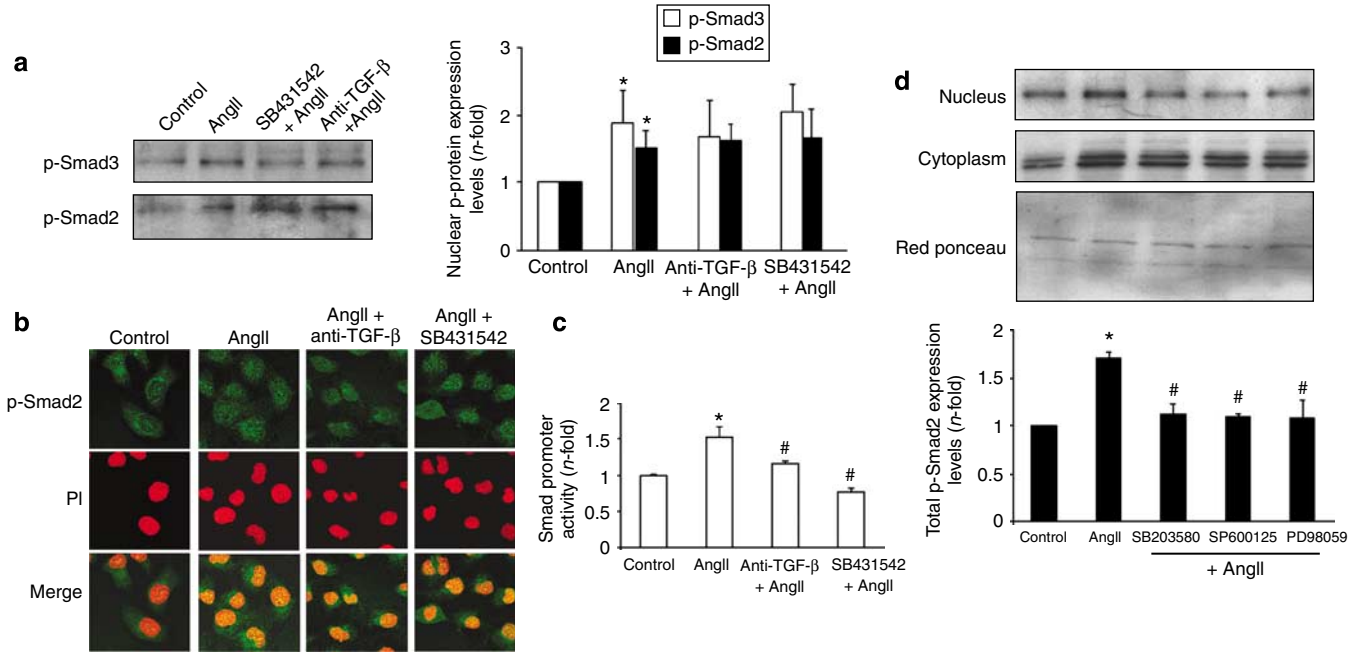

Figure 5 | AngII activates early Smad pathway independently of TGF-β in human tubuloepithelial cells. TGF-β was blocked by pretreatment with 10 μg/ml TGF-β neutralizing antibody or 10^{-5} mol/l SB431542 for 1 h, before AngII (10^{-7} mol/l) stimulation for 15 min. (**a**) Nuclear protein levels of phosphorylated-Smad2/3 were quantified by western blot. The figure shows a representative experiment on the right panel and data as mean ± s.e.m. of three experiments on the left. (**b**) The p-Smad2 was located by indirect immunofluorescence with a fluorescein isothiocyanate-labeled secondary antibody (green staining). The figure is representative of three experiments. (**c**) AngII-induced long-term Smad activation is mediated by TGF-β. HK2 cells were transfected with Smad/luc promoter and TK-renilla. After 24 h serum-starvation, TGF-β was blocked and cells were stimulated with AngII for 24 h. Then luciferase/renilla activity was measured. Data of mean ± s.e.m. of four experiments done in triplicate is shown. (**d**) MAPK activation is involved in AngII-induced Smad activation. HK2 cells were pretreated with 10^{-6} mol/l SB203580 (p38 inhibitor), 10^{-5} mol/l PD98059 (ERK p42/44 inhibitor), or 10^{-5} mol/l SP600125 (JNK inhibitor) for 1 h, before treatment with 10^{-7} mol/l AngII for 15 min. Nuclear and cytosolic fractions were extracted and levels of p-Smad2 were evaluated by western blot. Red ponceau staining was used as loading control. Panel (**d**) shows a representative experiment in the top panel and data of mean ± s.e.m. of three experiments in bottom panel. *$P<0.05$ vs control. #$P<0.05$ vs AngII.

Role of endogenous TGF-β on AngII-induced EMT

TGF-β is a mediator of AngII-induced fibrosis. The effects of AngII and TGF-β at 24 h on the regulation of EMT markers were similar (Figure 6). We therefore tested whether early AngII-induced EMT was TGF-β independent by evaluating vimentin expression. In HK2 cells, vimentin induction caused by AngII after 18 and 24 h of incubation was not diminished by the endogenous blockade of TGF-β (Figure 7a), suggesting that the initial induction of EMT is independent of TGF-β. However, TGF-β blockers significantly diminished AngII-induced vimentin expression after 3 days, as well as the phenotypic conversion to fibroblast-like morphology (Figure 7b and c), suggesting that TGF-β contributes to the progression of the EMT process caused by AngII.

AngII-induced EMT is mediated by Smad activation

To block Smad actions, cells were transiently transfected with a Smad7 expression vector that inhibits TGF-β/Smad-mediated transcriptional effects by interfering with receptor-mediated activation of R-Smad.[14] HK2 cells transfected with Smad7 markedly diminished EMT caused by AngII and TGF-β. Overexpression of Smad7 inhibited vimentin upregulation caused by both AngII and TGF-β after 24 h, compared with cells transfected with an empty vector, pcDNA3B (western blot; Figure 8a). By immunofluorescence, we observed that Smad7 overexpression impaired AngII-mediated changes in cell shape and induction of α-SMA staining after 3 days (Figure 8b), as observed with TGF-β, whereas cells transfected with pcDNA3B undergo EMT in response to both stimuli.

DISCUSSION

We have evaluated the effect of AngII on the Smad signaling pathway in the kidney. Our *in vivo* studies using the model of systemic infusion of AngII showed the activation of the Smad pathway as early as 24 h and remained elevated until 2 weeks. AngII-treated rats presented increased renal levels of phosphorylated Smad2/3 proteins and active Smad complexes, mainly in tubuloepithelial cells and in some glomerular cells. In cultured human tubuloepithelial cells, AngII caused a rapid activation of Smad pathway (observed at 15 min of incubation), showed by increased Smad2/3 phosphorylation, nuclear translocation of phosphorylated Smad2 and Smad4, and increased DNA-binding activity to CAGA-box oligonucleotide. Our data demonstrate that AngII

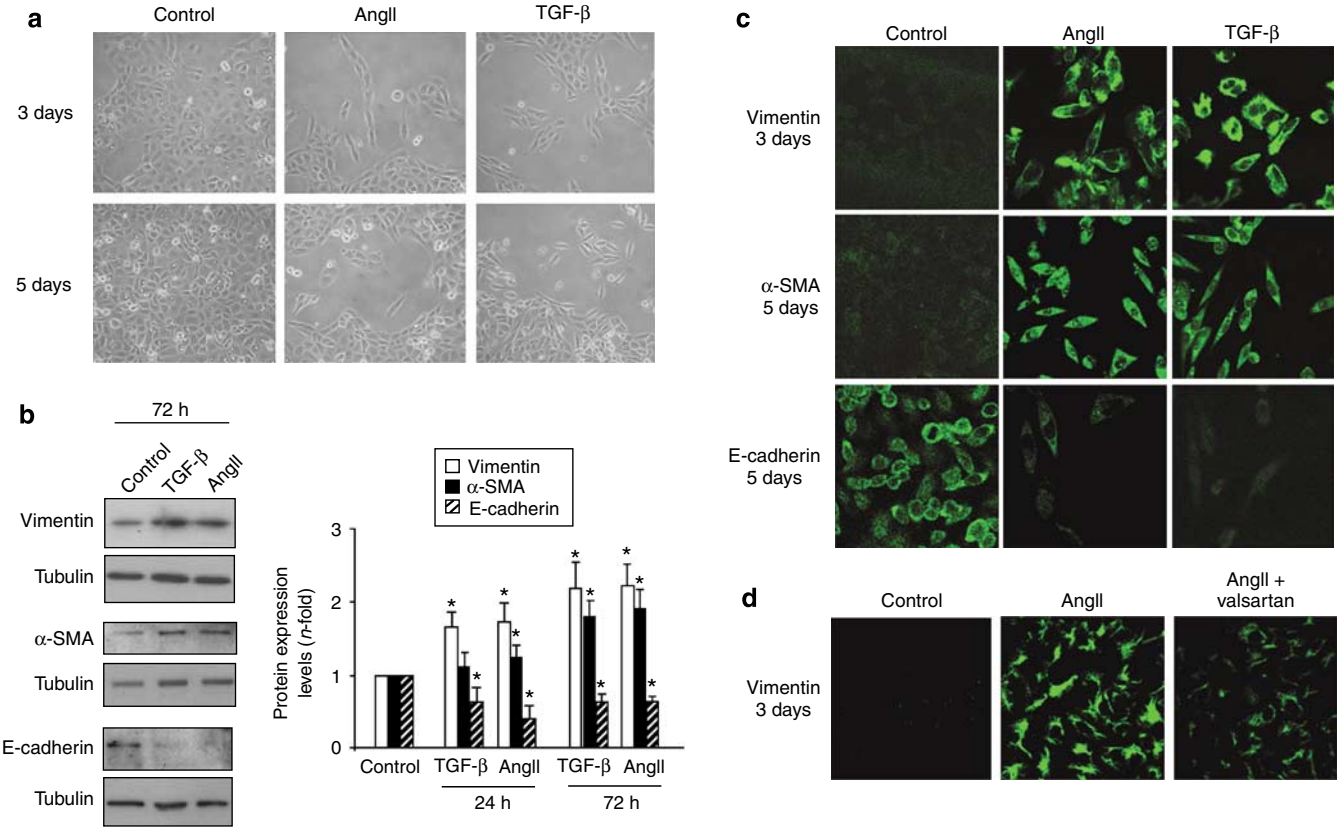

Figure 6 | AngII causes EMT in human tubuloepithelial cells. (**a**) Cells were stimulated with 10^{-7} mol/l AngII or 1 ng/ml of TGF-β1 for 3 and 5 days. Phase-contrast images were taken at ×200 original magnification. (**b**) AngII induces *de novo* expression of vimentin and α-SMA and the loss of E-cadherin. HK2 cells were treated with 10^{-7} mol/l AngII or 1 ng/ml of TGF-β1 for 24 and 72 h. Results of total protein expression were obtained from densitometric analysis and expressed as ratio protein/tubuline as *n*-fold over control. The figure shows a representative western blot in the left panel and data of total protein levels as mean ± s.e.m. of four independent experiments in the right panel. $^*P < 0.05$ vs control. (**c**) Confocal microscopy analysis. The vimentin, E-cadherin, and α-SMA were detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody. The figure is representative of three experiments. AngII induces vimentin expression via AT_1 receptor. Cells were pretreated with valsartan (10^{-6} mol/l) for 1 h and then treated with AngII for 24 h or 3 days. Figure (**d**) shows a representative confocal experiment of 3 done.

activates the Smad signaling in the kidney *in vivo* and in cultured human tubuloepithelial cells.

In the kidney, there is an interesting link between AngII and TGF-β. Angiotensin-converting enzyme inhibitors or AT receptor antagonists diminish renal expression of TGF-β and fibrosis.[3] In cultured renal cells, AngII stimulates TGF-β expression, and the blockade of TGF-β diminishes some AngII responses, including extracellular matrix regulation.[2,3] Infusion of AngII into rats also increased renal TGF-β synthesis.[24] However, we have found that AngII-induced Smad activation (observed at 24 h) occurs earlier than TGF-β upregulation (observed after 72 h in gene expression and later on in protein levels). These data suggest that in the kidney AngII causes a rapid activation of the Smad pathway by a TGF-β-independent mechanism. To further demonstrate whether the AngII effect was independent of endogenous TGF-β production, *in vitro* studies were done. Firstly, we have compared the time-course response of Smad activation and TGF-β induction. In tubuloepithelial cells, AngII only increases TGF-β production after 24 h of incubation (data not shown), whereas Smad activation was found after 15 minutes of AngII stimulation. Secondly, we blocked TGF-β using a neutralizing antibody against active TGF-β and an inhibitor of TGF-β receptor. The blockade of endogenous TGF-β did not alter nuclear translocation of phosphorylated Smad2 and Smad4 and Smad DNA-binding activity observed after 15 min of incubation with AngII, showing a TGF-β-independent Smad signaling activation. However, TGF-β mediates late activation of Smad pathway, because TGF-β blockers diminished AngII-induced Smad-dependent transcription noted after 24 h. Similar findings have been described in VSMCs. The early AngII-induced Smad activation is TGF-β independent,[10] whereas the late effect is mediated by TGF-β.[21]

There are several Smads proteins. Smad2 and Smad3 are specific mediators of TGF-β/activin pathways, whereas Smad1, Smad5, and Smad8 are involved in bone morphogenetic protein signaling.[6] Smad3 is critical for EMT in models of kidney injury and in aggressive carcinoma metastasis.[5,14] Smad3 and Smad2 have different roles in

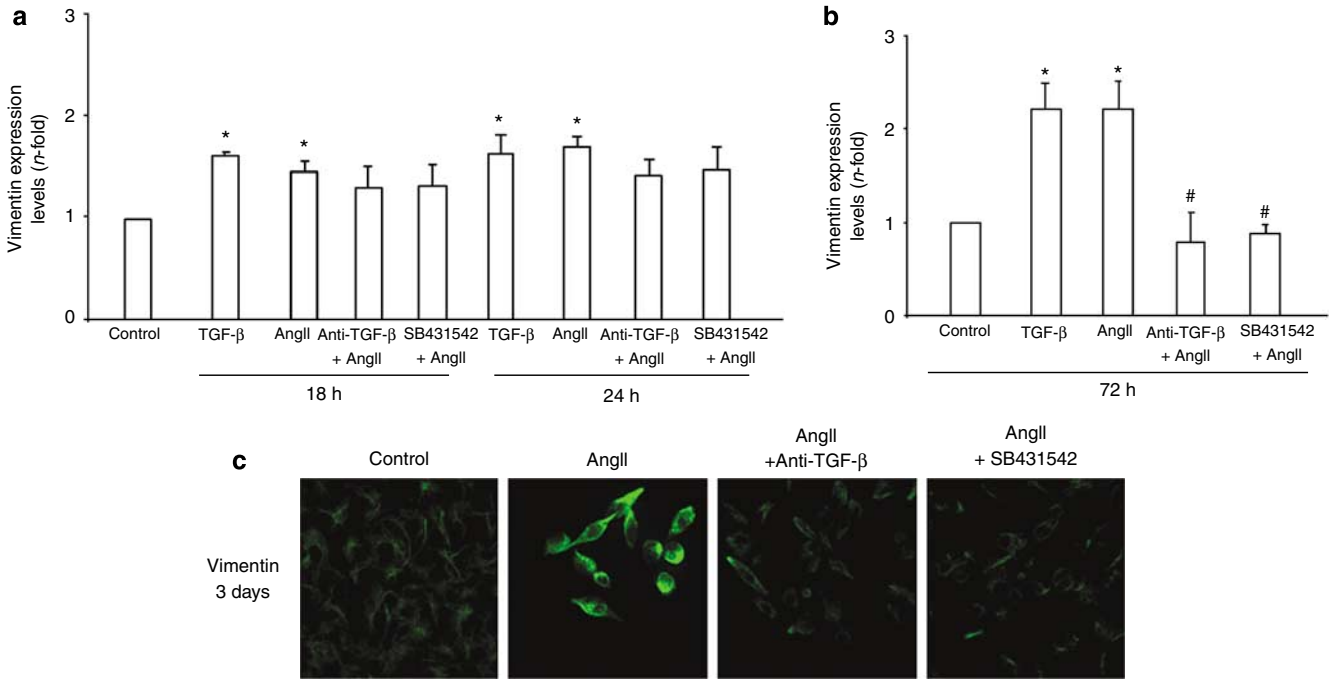

Figure 7 | AngII activates early EMT markers independently of TGF-β in tubuloepithelial cells. After TGF-β blockade, cells were stimulated for 18 and 24 h. (**a**) Vimentin expression as mean ± s.e.m. of three western blot experiments. TGF-β is involved in EMT and morphological changes observed after 3 days of AngII treatment. (**b**) Vimentin expression as mean ± s.e.m. of three western blot experiments. *$P<0.05$ vs control. #$P<0.05$ vs AngII. (**c**) Three representative inmunofluorescense experiments done.

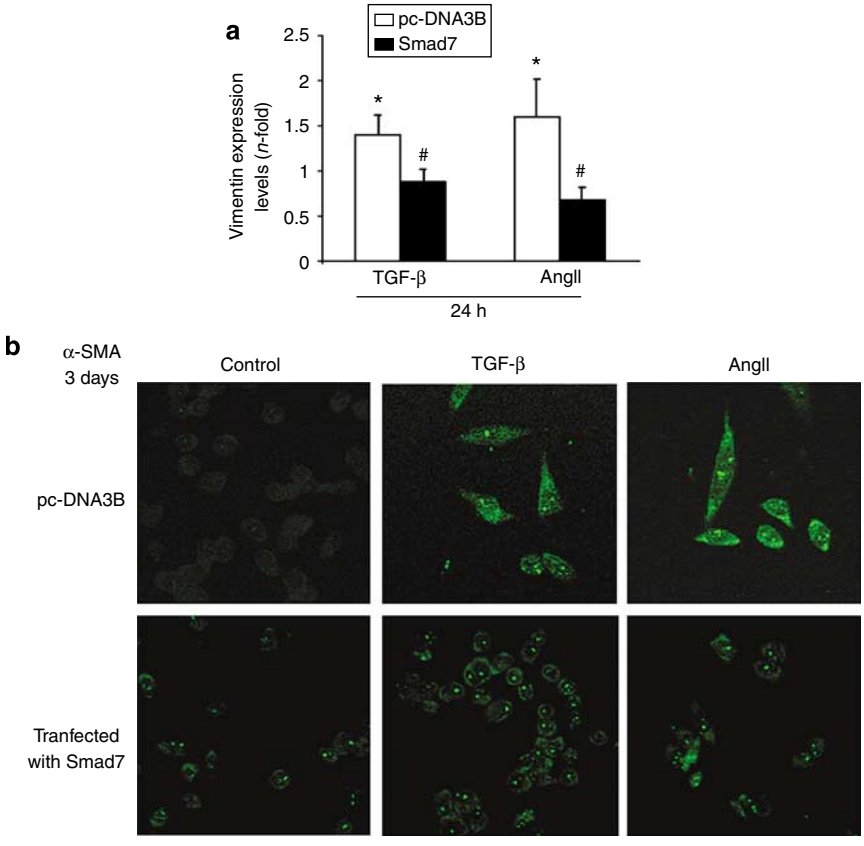

Figure 8 | Smad is involved in AngII-induced EMT. HK2 cells were transiently transfected with Smad7 expression vector or empty vector (pcDNA3B). Then, cells were stimulated with AngII or TGF-β for (**a**) 24 h and (**b**) 3 days (**b**). (**a**) Data of total vimentin protein levels as mean ± s.e.m. of three independent experiments. *$P<0.05$ vs control; #$P<0.05$ vs empty vector. (**b**) The α-SMA was detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody. The figure is representative of three experiments done.

TGF-β-induced EMT. The regulation of connective tissue growth factor (CTGF) and E-cadherin expression are Smad3 dependent, whereas MMP-2 is regulated by Smad2. Both Smad2 and Smad3 regulate α-SMA.[5] In VSMCs, Smad3 is involved in AngII-mediated vascular fibrosis.[21] Our data show that AngII activates Smad2/3 proteins in the kidney and cultured renal cells, showing a similar pathway than TGF-β. There is a cross-talk between MAPKs and Smad.[6] Overexpression of constitutively active members of the ras/MEK/ERK cascade promotes Smad3-dependent processes in kidney mesangial cells, while blocking the nuclear accumulation of Smads in epithelial cells.[25] In VSMC, AngII activates Smad pathway via MAPKs activation.[10,21] In HK2 cells, we have found that all three MAPK inhibitors (p38, ERK, and JNK) significantly diminished AngII-induced Smad2 phosphorylation. Future studies are needed to elucidate the interactions between Smad and MAPK signaling *in vivo* in the kidney.

Rats that have been AngII-infused for 2 weeks presented mild tubular damage, as described,[16,17] and induction of EMT, shown by α-SMA upregulation and downregulation of E-cadherin. Several data in experimental models of renal injury also indicate that AngII participates *in vivo* in the EMT process.[16] Evidences of EMT have also been reported in human diabetic and nondiabetic progressive nephropathies. In these renal pathologies, upregulation of renal renin–angiotensin system and tubular myofibroblast activation have been described.[18,26] We have demonstrated that, in cultured human tubuloepithelial cells, AngII causes suppression of E-cadherin and *de novo* vimentin and α-SMA expression, leading to the loss of epithelial cell adhesion and the change from epithelial to fibroblast-like morphology, suggesting a transitional phase in the dynamic phenomenon of EMT, confirming previous data.[15] TGF-β is a key factor in renal EMT, by initiating and regulating the entire process.[7,11,27] We have compared the effect of AngII with that of TGF-β. After 24 h of incubation, AngII induced vimentin and α-SMA production and suppression of E-cadherin, showing a similar early regulation of EMT markers to that observed with TGF-β. The presence of fibroblast-like cells was found at 3 days, with more positive cells at 5 days, but without differences between AngII and TGF-β, suggesting that both factors have a similar effect on EMT. We have further investigated whether TGF-β mediates AngII-induced EMT. In human tubuloepithelial cells, the induction of vimentin production at 18 h of incubation with AngII was not diminished by TGF-β blockers, showing that early EMT caused by AngII is TGF-β independent. However, the TGF-β blockade diminished the phenotypic conversion to fibroblast-like morphology and induction of vimentin after 3 days, showing that endogenous TGF-β production participates in long-term EMT. Systemic AngII infusion elicited changes in EMT markers after 2 weeks. At this time, Smad activation and TGF-β upregulation were also found, indicating that, in chronic infusion of AngII, the activation of Smad pathway by endogenous TGF-β could contribute to renal damage progression.

Transforming growth factor-β promotes EMT by several mechanisms, with Smad pathway being the most relevant. Our *in vitro* studies, blocking the Smad pathway by transient transfection with Smad7, which interfere with activation of Smad2 and Smad3, demonstrate that Smad pathway participates in AngII-induced EMT. Thus, in cultured tubuloepithelial cells, Smad7 overexpression prevented vimentin and α-SMA induction and the transition to fibroblast-like morphology caused by AngII. In different animal models, including unilateral ureteral obstruction and experimental hypertension, Smad7 overexpression attenuates renal fibrosis.[28] The same has been observed in animal models of peritoneal fibrosis induced by peritoneal dialysis, where Smad7 overexpression inhibited fibrosis.[29,30] Different therapies to interfere with TGF-β, including neutralizing antibodies, antisense oligonucleotides, and decorin, have been shown to diminish renal fibrosis. However, these treatments cannot be used in humans. Renin–angiotensin system blockers are commonly used in human renal diseases with proven end-organ protective effects.[3] The use of AngII blockers, besides inhibiting AngII actions, also interfere with TGF-β/Smad signaling, providing an important tool to hinder TGF-β and prevent the loss of functional renal tissue.

Our results show that AngII activates the Smad signaling system by a TGF-β-independent process in the kidney, both *in vivo* and *in vitro*. We have also found that Smad proteins participate in AngII-induced EMT. This novel finding suggests that Smad activation could participate in AngII-mediated profibrogenic effects in renal diseases.

MATERIALS AND METHODS

Experimental studies

Systemic infusion of AngII (in saline) was done into male Wistar rats of 3 months of age (subcutaneous osmotic minipumps, Alza Corp., CA), at 100 ng/kg/min for different times (from 24 h to 2 weeks; $n = 4$–8 animals per group). A control group of saline-infused rats of the same age was also studied ($n = 8$ animals). The kidneys were perfused *in situ* with cold saline before removal. One kidney was fixed, embedded in paraffin, and used for immunohistochemistry, and the other kidney was snap-frozen in liquid nitrogen for RNA and protein studies (done in renal cortex). All experimental procedures were approved by the Animal Care and Use Committee of our Institution, according to the guidelines for ethical care of the European Community.

Cell cultures

HK2 cells were grown in RPMI with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin, ITS, and hydrocortisone in 5% CO_2 at 37°C. At 60–70% of confluence, the cells were growth-arrested in a serum-free medium for 24 h before the experiments. AngII (10^{-7} mol/l) was added each day, and the medium and all stimuli were replaced every 48 h. Cell culture reagents were obtained from Life Technologies Inc. and AngII was obtained from Bachem. The inhibitors used were PD98059, ERK1/2 inhibitor, SB203580, p38 MAPK inhibitor, and SP600125, JNK-1,-2,-3 inhibitor from Stressgen Bioreagents Corp. (Victoria, BC, Canada). None of the inhibitors were toxic at the doses used (evaluated by cell viability assay microculture tetrazoluin

assay, Promega). Antibodies employed were α-SMA (Sigma Chemical Co., St Louis, MO, USA); vimentin (BD Pharmingen, San Diego, CA, USA), E-cadherin (R&D Systems, Minneapolis, MN, USA), Smad2, Smad4, phosphorylated Smad2/3 (Sta. Cruz Biotechnology, Sta. Cruz, CA, USA), pSmad2 (Cell Signaling Technology, Danvers, MA, USA), pSmad3 kindly donated by Dr Leof (Mayo Clinic, Baltimore), TGF-β (ABCam, Cambridge, UK), and secondary antibodies (Amersham, Buckinghamshire, UK).

Protein studies

To quantify protein levels, western blot was done.[10,31] Protein content was determined by BCA method (Pierce Biotechnology, Rockford, IL, USA). The efficacy of protein loading and transfer to membranes was assessed by α-tubulin, GAPDH, and Ponceau S staining.

Immunocytochemistry was performed in cells growing in coverslips, fixed, treated with 0.1% Triton X-100, incubated with primary antibodies, followed by fluorescein isothiocyanate-conjugated antibodies.[10] Nuclei were stained with 1 μg/ml propidium iodide. Samples were mounted in Mowiol 40–88 and examined by a laser scanning confocal microscope (Leika).

Immunohistochemistry was done in paraffin-embedded renal sections.[18] Renal sections of 4 μm were deparaffinized, rehydrated, their endogenous peroxidase was blocked and incubated with primary antibody (overnight, 4°C), followed by incubation with the corresponding secondary antibody, and revealed by standard techniques. The specificity was checked by the omission of primary antibody and use of nonimmune sera (not shown).

Gene studies

Total RNA was isolated with Trizol (Gibco, Invitrogen, Carlsbad, CA, USA) and gene expression was analyzed by real-time PCR, performed on an ABI Prism 7500 sequence detection PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Assay IDs used are TGF-β:Rn00572010_m1, thromsbospondin-1: Rn01513693_m1, Smad4;Rn00570593_m1, Smad7; Rn00578319_m1, α-SMA:Rn00570060_g1, E-cadherin:Rn00580109_m1, and vimentin:Rn0059738_m1. For normalized data, different approaches were done using several housekeeping genes, including GAPDH, Histone-3, and 18s ribosomal RNA expression (assay IDs: Rn99999916_m1 and Hs99999901_s). Each animal was evaluated independently by duplicate, and data were expressed as mean ± s.e.m. of eight animals per group as *n*-fold increase vs control group.

Analysis of Smad DNA-binding activity

Smad DNA-binding activity was determined in 6 μg nuclear extracts as described,[10] by binding with radioactive labeled consensus CAGA-box oligonucleotide (5′-TCGAGAGCCAGACAAAAAGCCA GACATTTAGCCAGACAC-3′, Sta. Cruz), and complexes were analyzed by electrophoretic mobility shift assay. Competition assays were done with a 100-fold excess of unlabeled or mutant oligonucleotide (5′-TCGAGAGCTAGATAAAAAGCTAGATATTAGCTAG ATAC-3).

Southwestern histochemistry was used for Smad detection as described,[18] including controls and the Smad binding consensus sequence (5′-GAGTATGTCTAGACTGACAATGTAC-3′).

Transfection, DNA constructs, and promoter studies

HK2 cells in fetal bovine serum were transiently transfected for 18 h with FuGENE (Roche Molecular Biochemicals, Indianapolis, IN, USA) and PcDNA3-FLAG-Smad7 expression vector (kindly donated by Dr Massagué, Memorial Sloan-Keternig Cancer Center, New York, USA) or empty vector (pcDNA3B). Cells were growth-arrested for 24 h before experiments. To demonstrate Smad7 transfection efficacy, an anti-FLAG antibody was used (not shown). Smad-dependent promoter activation was evaluated by transfection of Smad/luc (kindly donated by Dr Volgestein, Baltimore, MD, USA) and TK-renilla as internal control, as described.[10]

Statistical analysis

Histochemistry was quantified by image analysis using a KZ 300 imaging system 3.0. (Zeiss, Munchen-Hallbergmoos, Germany). For immunohistochemistry, the percentage of the stained area was calculated as the ratio of stained area vs total field area, and staining score is expressed as density/mm^2. For Southwestern histochemistry, positive cells were counted and expressed as number of Smad positive cells/mm^2. For each sample, the mean data was obtained by analysis of 20 different fields (× 200). These experiments were performed in two kidney sections per experimental animal to obtain a mean score for each of them. In all cases, evaluations were performed by two independent observers in a blinded fashion, and the mean score value was calculated for each rat.

The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad, Hercules, CA, USA). Results are expressed as *n*-fold increase over control as mean ± s.e.m. Equality of variances was tested with Levene's test. Normally distributed continuous variables with equal variances were analyzed with analysis of variance, otherwise with Kruskall–Wallis test. A P value <0.05 was considered significant. Tests were done using the SPSS 11.5 software package.

DISCLOSURE

The authors declared no competing interests.

ACKNOWLEDGMENTS

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2. Modulación farmacológica de la transición epitelio-mesenquimal causada por Angiotensina II. Papel de las rutas de señalización ROCK y MAPKs.

Continuando con el estudio de los mecanismos implicados en la TEM causada por AngII, nuestro siguiente objetivo ha sido evaluar la participación de la activación de proteínas quinasas en este proceso. Para ello se han realizado los experimentos presentados en este trabajo.

En células túbulo-epiteliales humanas en cultivo observamos que el bloqueo de la cascada de las MAPKs, utilizando inhibidores específicos de p38 (con SB203580), ERK (quinasa reguladora de señales extracelulares 1/2; con PD98059) y JNK (quinasa Jun N-terminal; con SP600125), previene de la conversión fenotípica de estas células epiteliales a miofibroblastos. El bloqueo de la ruta RhoA/quinasa de Rho, mediante la transfección de un vector dominante negativo de RhoA o mediante la inhibición de ROCK con Y-27632 o fasudil, inhibió la TEM causada por AngII.

Otro objetivo desarrollado en este trabajo ha sido evaluar si el factor profibrótico CTGF regula la TEM causada por AngII. En este estudio hemos observado que en células túbulo-epiteliales humanas los inhibidores de las tres MAPKs (p38, ERK y JNK) y de ROCK previenen la sobre-expresión de CTGF inducida por AngII. El bloqueo de la producción endógena de CTGF, mediante oligonucleótidos antisentido, inhibió la TEM inducida por AngII a tiempos cortos y largos, lo que indica que CTGF podría actuar como un mediador de los efectos fibróticos de AngII en el riñón. Estos datos sugieren que el bloqueo del CTGF endógeno, podría ser una buena alternativa terapéutica para las enfermedades renales crónicas.

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Research Paper

Pharmacological Modulation of Epithelial Mesenchymal Transition Caused by Angiotensin II. Role of ROCK and MAPK Pathways

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Purpose. Tubulointerstitial fibrosis is a final common pathway to end-stage chronic kidney diseases, which are characterized by elevated renal angiotensin II (AngII) production. This peptide participates in kidney damage inducing fibrosis and epithelial mesenchymal transition (EMT). Our aim was to describe potential therapeutic targets in AngII-induced EMT, investigating the blockade of different intracellular pathways.
Methods. Studies were done in human tubular epithelial cells (HK2 cell line), evaluating changes in phenotype and EMT markers (Western blot and immunofluorescence).
Results. Treatment of HK2 cells with AngII for 3 days caused transdifferentiation into myofibroblast-like cells. The blockade of MAPKs cascade, using specific inhibitors of p38 (SB203580), extracellular signal-regulated kinase1/2 (ERK; PD98059) and Jun N-terminal kinase (JNK) (SP600125), diminished AngII-induced EMT. The blockade of RhoA/ROCK pathway, by transfection of a RhoA dominant-negative vector or by ROCK inhibition with Y-27632 or fasudil, inhibited EMT caused by AngII. Connective tissue growth factor (CTGF) is a downstream mediator of AngII-induced EMT. MAPKs and ROCK inhibitors blocked CTGF overexpression induced by AngII. HMG-CoA reductase inhibitors, although blocked AngII-mediated kinases activation, only partially diminished EMT and did not regulate CTGF.
Conclusions. These data suggest a potential therapeutic use of kinase inhibitors in renal fibrosis.

KEY WORDS: angiotensin; epithelial mesenchymal transition; kinase inhibitors; renal damage; statins.

INTRODUCTION

The incidence of renal diseases is growing in Western countries. Independently of the initial insult a common feature of renal diseases is the progression to tubulointerstitial fibrosis and end-stage kidney failure. Among the current clinical treatments, the blockade of angiotensin II (AngII) is one of the best pharmacological options with proven organ-protective effects (1). However, these drugs only slow the progression of the disease and novel therapeutic options are needed to regress renal fibrosis. The molecular mechanisms involved in renal fibrosis and its pharmacological modulation are very important fields of research in chronic kidney diseases.

Tubulointerstitial fibrosis is characterized by an excessive accumulation of extracellular matrix proteins, such as collagens, in part attributable to an elevated synthesis mainly by interstitial fibroblasts. Many evidences suggest that under pathological conditions renal tubuloepithelial cells can undergo epithelial mesenchymal transition (EMT) becoming matrix-producing fibroblasts, and therefore contribute to renal fibrosis and progression to end-stage kidney disease (2). EMT is characterized by a phenotypic conversion from epithelial cells to fibroblast-like morphology. During this process, there is an induction of mesenchymal markers, such as α-smooth muscle actin (α-SMA) and vimentin, and epithelial markers disappear, like E-cadherin that is essential for the structural integrity of renal epithelium (2). Most of the studies of EMT have focused on TGF-β responses. This growth factor participates in all of the steps of EMT (2). AngII shares many cellular responses with TGF-β (3, 4). In the kidney, AngII actively participates in renal fibrosis, in part mediated by TGFβ (5). Recently, we

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ABBREVIATIONS: AngII, angiotensin II; AT, angiotensin receptors; CTGF, connective tissue growth factor; EMT, epithelial mesenchymal transition; ERK, extracellular signal-regulated kinase1/2; FBS, fetal bovine serum; HK2, human tubular epithelial cell line; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; JNK, Jun N-terminal; MAPK, mitogen activated kinases; ROCK, rho-kinase; TGF-β, transforming growth factor-beta; VSMC, vascular smooth muscle cells.

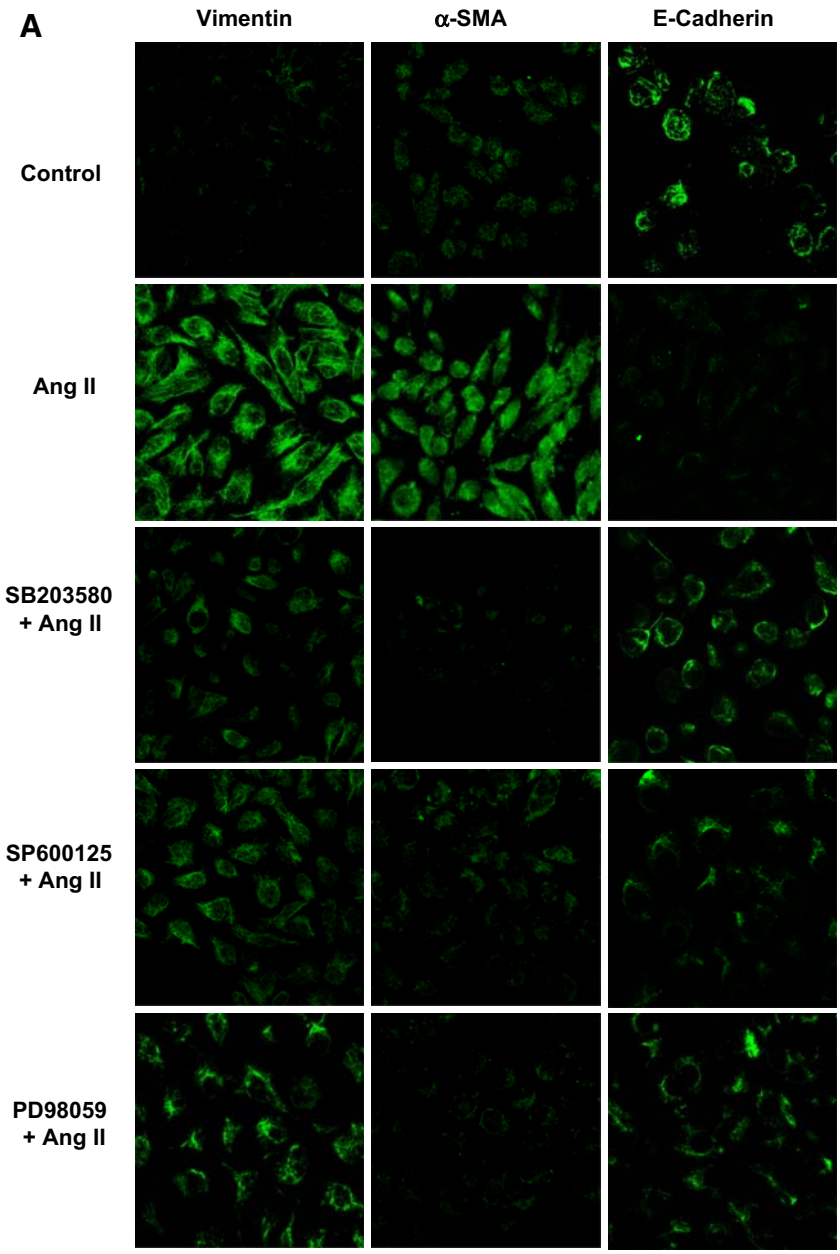
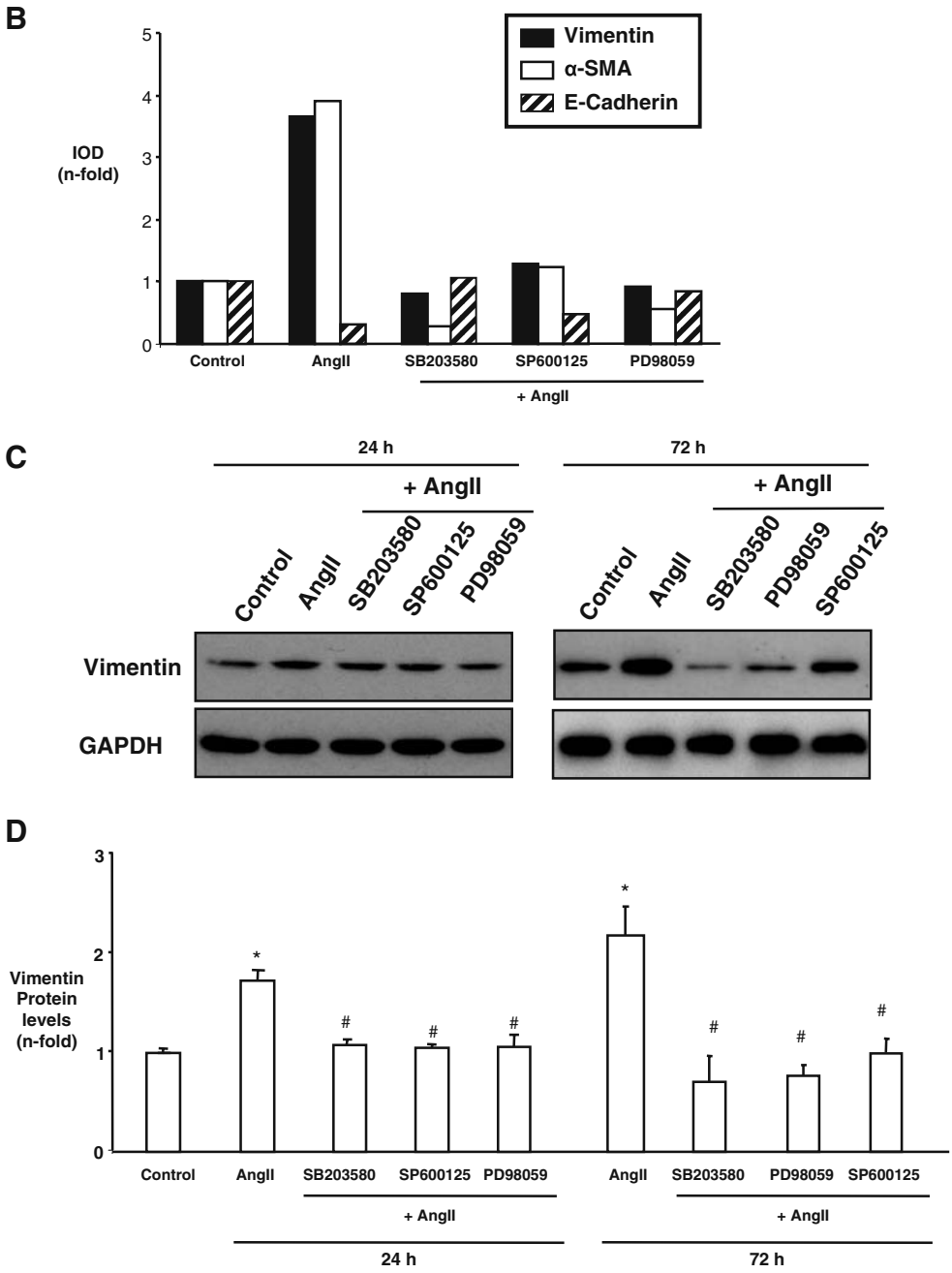


Fig. 1. MAPKs inhibitors diminish AngII-induced EMT in human tubuloepithelial cells. Cells were preincubated for 1 h with the following MAPKs inhibitors: SB203580 (p38 inhibitor, at 10^{-6} mol/l), PD98059 (ERK p42/44 inhibitor, at 10^{-5} mol/l) and SP600125 (JNK inhibitor, at 10^{-5} mol/l) and then treated with 10^{-7} mol/l AngII for 3 days. **A** Vimentin, α-SMA and E-Cadherin were detected by an indirect immunostaining using FICT-labeled secondary antibodies, and evaluated by confocal microscopy. Figure shows a representative experiment of three done. All three MAPKs inhibitors markedly diminished the phenotypic conversion caused by AngII; the cells present an epithelial morphology with positive E-cadherin staining, but a weak immunostaining for vimentin and α-SMA. **B** Quantification of immunofluorescence data expressed as integrated optical density (IOD) as described in Methods. Vimentin expression was quantified by Western blot after 24 and 72 h of incubation. **C** A representative Western blot and **D** data as mean ± SEM of three independent experiments. *$P<0.05$ *vs* control. #$P<0.05$ *vs* AngII.

have shown that AngII directly activates the Smad signaling system in the kidney and induces EMT through TGF-β/Smad pathway (6). Many studies have shown that AngII inhibitors diminish renal TGF-β overproduction and signaling activation (3, 4), showing that these drugs are one of the best options to block TGF-β in humans.

AngII binds to specific receptors, AT_1 and AT_2, to activate cellular responses. AT_1 receptor mediates upregula-

Fig. 1. (continued)



tion of growth factors, extracellular matrix accumulation and EMT (5,6). The AT_1 signaling mechanisms are similar to those activated by cytokines, and include activation of protein kinases, as for example mitogen-activated protein kinase (MAPK) cascade and Rho-kinase (ROCK) (5). Several intracellular signaling systems are involved in EMT and renal fibrosis. Recent studies have demonstrated that the MAPK pathway regulates EMT caused by TGF-β (7, 8). Activation of small Rho GTPases is a key step in EMT (9). ROCK is a downstream target of RhoA involved in TGF-β-mediated EMT (10). For this reason, we have investigated the potential role of MAPK and RhoA/ROCK pathways in AngII-induced EMT.

Several clinical trials have demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) exert beneficial effects in patients at high risk of developing cardiovascular events (11). These drugs combine hypolipemic effect with other effects, such as antioxidant, anti-inflammatory, immunomodulatory, and antithrombotic (called "pleiotropic" effects) (11, 12). Their beneficial actions can be attributed to the inhibition of intracellular signaling pathways, including MAPK and RhoA/ROCK pathways (11–13). However, the role of statins in renal disease progression and in the regulation of renal fibrosis and EMT is not completely elucidated (14–17). In this work, we have investigated whether statins could directly modulate AngII-induced EMT, studying

the regulation of CTGF and the molecular mechanisms underlying this process, evaluating the role of the activation of Rho/ROCK and MAPK pathways. These experiments might help to unveil the mechanisms of renal damage perpetuation and suggest novel therapeutic strategies for the modulation of the pathobiology of renal injury.

MATERIALS AND METHODS

Cell Cultures

HK2 cells (human renal proximal tubuloepithelial cells) were grown in RPMI with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin, ITS (5 μg/ml) and hydrocortisone (36 ng/ml) in 5% CO_2 at 37°C. At 60–70% of confluence, cells were growth-arrested in serum-free medium for 24 h before the experiments.

Materials

AngII (from Fluka), at the dose of 10^{-7} mol/l, was added each day, and medium and all stimuli were replaced every 48 h. Cell culture reagents were obtained from Life Technologies, Inc. Atorvastatin was from Pfizer (Madrid, Spain) and simvastatin from Merck Sharp and Dome (Madrid). PD98059; ERK1/2 inhibitor, SB-203580; p38 MAPK inhibitor, and SP600125: JNK-1,-2,-3 inhibitor were from Stressgen Bioreagents Corp.(Victoria, British Columbia, Canada); Fasudil and Y-27632; ROCK inhibitors from Tocris Cookson (Bristol, UK), and the rest of compounds from Sigma-Aldrich. None of the inhibitors were toxic at the doses used (evaluated by cell viability assay MTS-PMS, Promega, not shown). The antibodies employed were: Smooth muscle α-actin (α-SMA) (Dako); Vimentin (BD Pharmingen), CTGF from Torrey Pines Biolabs (Houston, TX, USA), phospho-JNK1/2 from Stressgen Bioreagents Corp; Phospho-ERK1/2, ERK1/2, JNK1/2 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), GAPDH (Calbiochem), peroxidase-conjugated secondary antibodies (Amersham). To block CTGF actions, we used a CTGF antisense oligonucleotide, constructed with a 16 mer derived from the starting translation site, which contained the initial ATG whose sequence is 5′-TACTGG CGGCGGTCAT-′3.

Transfection and DNA Constructs

HK2 cells, in 24 well-plates, were transiently transfected with FuGENE (Roche Molecular Biochemicals) and the reporter expression vectors for 18 h. The expression vectors containing cDNAs for constitutively active RhoA (pcDNA3-Q63L-RhoA) and wild type RhoA (pcDNA3-wtRhoA) gifts from Dr. Piero Crespo (Instituto de Investigaciones Biomédicas,

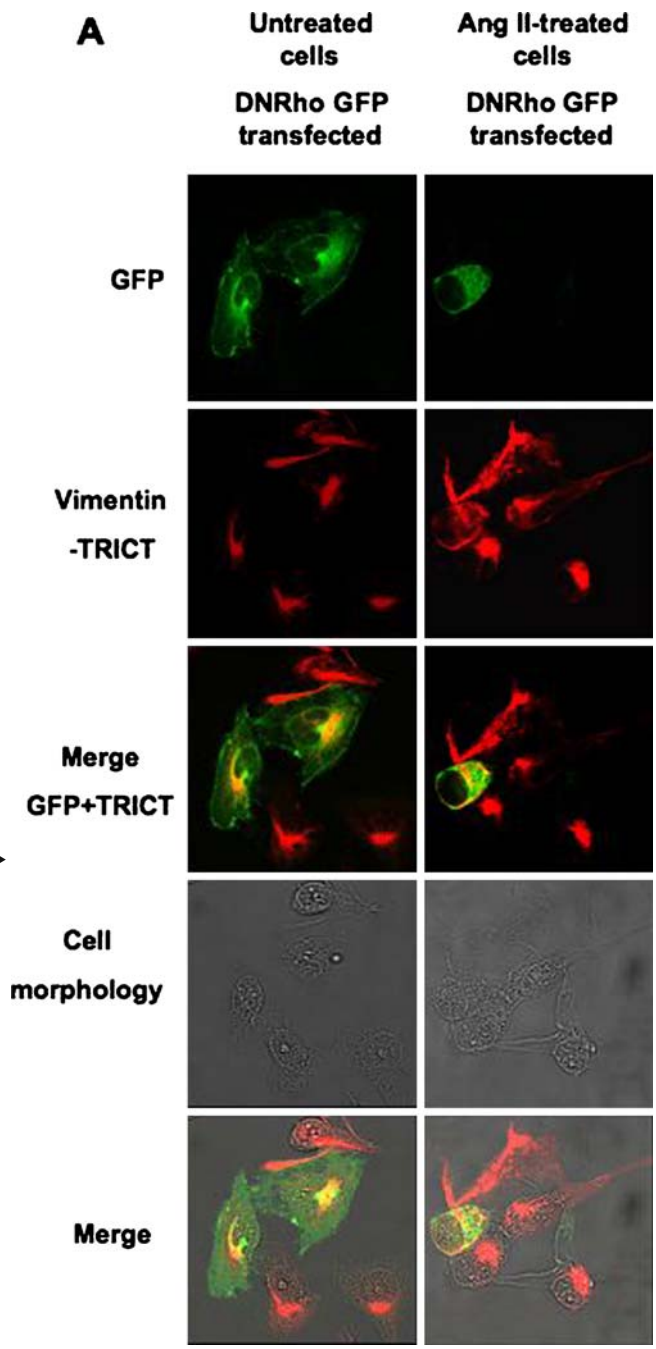
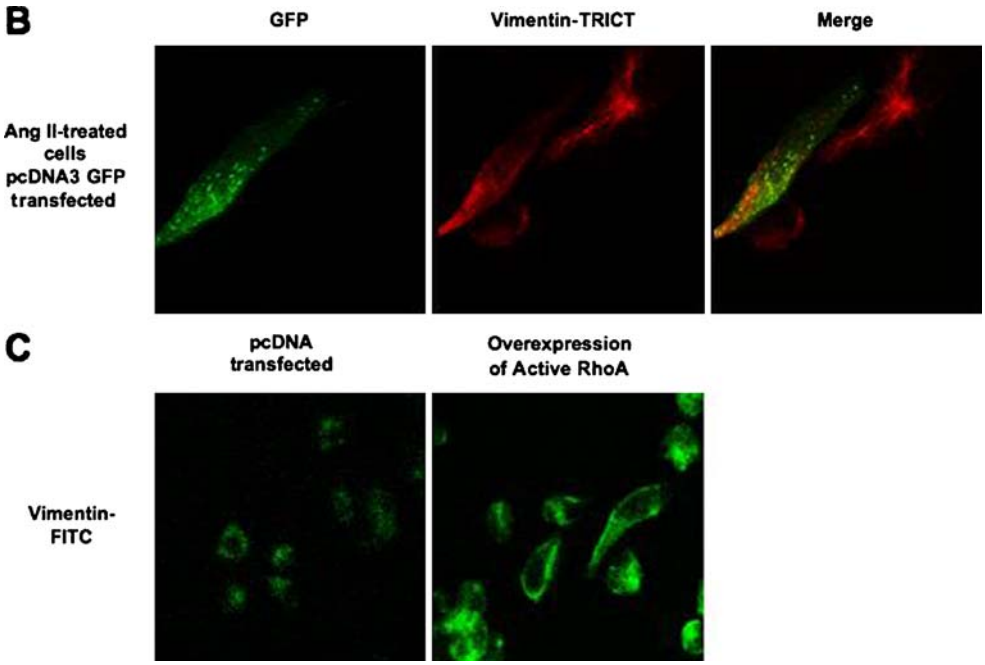


Fig. 2. The RhoA/ROCK pathway is involved in AngII-induced EMT in human tubuloepithelial cells. Cells were transiently transfected with **A** dominant negative isoform of RhoA (pcDNA3–GFP–N19RhoA) or **B** empty vector (pcDNA3–GFP) for 18 h and then treated with 10^{-7} mol/l AngII for 3 days. Immunocytochemistry shows green fluorescence in transfected cells (GFP-positive). Vimentin was detected by an indirect immunostaining using a TRICT-labeled secondary antibody (red staining). The colocalization of transfected cells and positive vimentin staining is shown by yellow staining (in merge GFP + TRICT). To better follow this data the morphology of the cells is shown. Merge: unstimulated cells present round shape, with a slight vimentin staining mainly in the nuclear membrane, showing no differences in cell shape and vimentin distribution between transfected (green) and non-transfected cells. **C** Cells were transiently transfected with a wild type of RhoA (WT–RhoA) and empty vector (pcDNA3B). Vimentin was detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody (green staining) after 48 h of transfection. The figures of confocal microscopy show a representative experiment of three done.

Fig. 2. (continued)



Madrid, Spain) and dominant-negative RhoA (pcDNA3-GFP-N19RhoA) and empty vector (pcDNA3B-GFP) from Dr. del Pozo (CNIC, Madrid, Spain). After transfection, cells were growth-arrested for 24 h before confocal microscopy experiments. In these experiments several differences in cell shape can be found compared to pharmacological studies, mainly due to the lower cell density necessary for a good efficacy of transfection and the different secondary antibody used.

Protein Studies

Cells were homogeneized in lysis buffer [170 mmol/l Tris HCl, 22% glycerol, 2,2% sodium dodecyl sulfate (SDS) with 0,1 mmol/l phenylmethylsulfonyl fluoride, NaF, dithiothreitol, ortovanadate and a protease inhibitor cocktail] and then separated by SDS-polyacrilamide gel electroforesis. CTGF, EMT markers and the phosphorylation levels of ERK and JNK levels were determined in total protein extracts by Western blot. Fifty micrograms of proteins were loaded in each lane. Protein content was determined by the BCA method (Pierce, Rockford, IL, USA). The efficacy of protein transfer to the membranes was assessed by Red Ponceau staining (not shown). Results of total protein expression were obtained from densitometric analysis and expressed as ratio protein/GAPDH or phosphorylated/total protein as *n*-fold over control.

For immunocytochemistry, cells growing in coverslips were fixed in merckofix (Merck), treated with 0.1% Triton-X100, incubated with primary antibodies followed by a FITC or TRIC-conjugated secondary antibody. The absence of primary antibody was the negative control. Samples were mounted in Mowiol 40–88 (Sigma) and examined by a laser scanning confocal microscope (Leika). The experiments were done with 3 different cell culture preparations. To validate the protein data obtained by Western blot and immunofluorescence, we have quantified the experiments of confocal microscopy using the Image-Pro plus 4.5.0.29 (Media Cybernetic Inc). The data are expressed as an arbitrary quantification of integrate optical density (IOD), calculated as average of density of fluorescence per area. These data are shown as n-fold of increase *vs* AngII of the representative experiment shown in the corresponding figure.

Quantification and Statistical Analysis

The autoradiographs were scanned using the GS-800 calibrated densitometer (Quantity One, Bio-Rad, Spain). Results are expressed as *n*-fold over control as mean ± SEM of experiments made. One-way ANOVA was used to test compare protein expression levels between groups. When statistical significance was found, Bonferroni post hoc comparison test was used to identify group differences. Differences were considered significant at $p<0.05$. Statistical analyses were conducted using the SPSS statistical software, version 11.0 (SPSS).

RESULTS

MAPKs Inhibitors Diminish AngII-Induced EMT in Human Tubuloepithelial Cells

In human tubuloepithelial cells (HK2 cell line) incubation with AngII for 3 days causes a phenotypic conversion from epithelial cells to myofibroblast-like cells, as described (6,18). The transformed cells lost the typical cobblestone pattern of an epithelial monolayer, as well as the expression

of the epithelial marker E-cadherin, and displayed a spindle-shape, fibroblast-like morphology associated with the induction of the mesenchymal markers, vimentin and α-SMA, which are not found in unstimulated epithelial cells (Fig. 1A).

We have investigated the involvement of MAPKs cascade by a pharmacological approach, using specific inhibitors of p38 (SB203580), extracellular signal-regulated kinase1/2 (ERK; PD98059) and Jun N-terminal kinase (JNK; SP600125) (19, 20). We found that all three MAPKs inhibitors prevented the AngII-induced phenotypic conversion into myofibroblasts observed after 3 days of treatment, and markedly diminished the presence of vimentin and α-SMA-

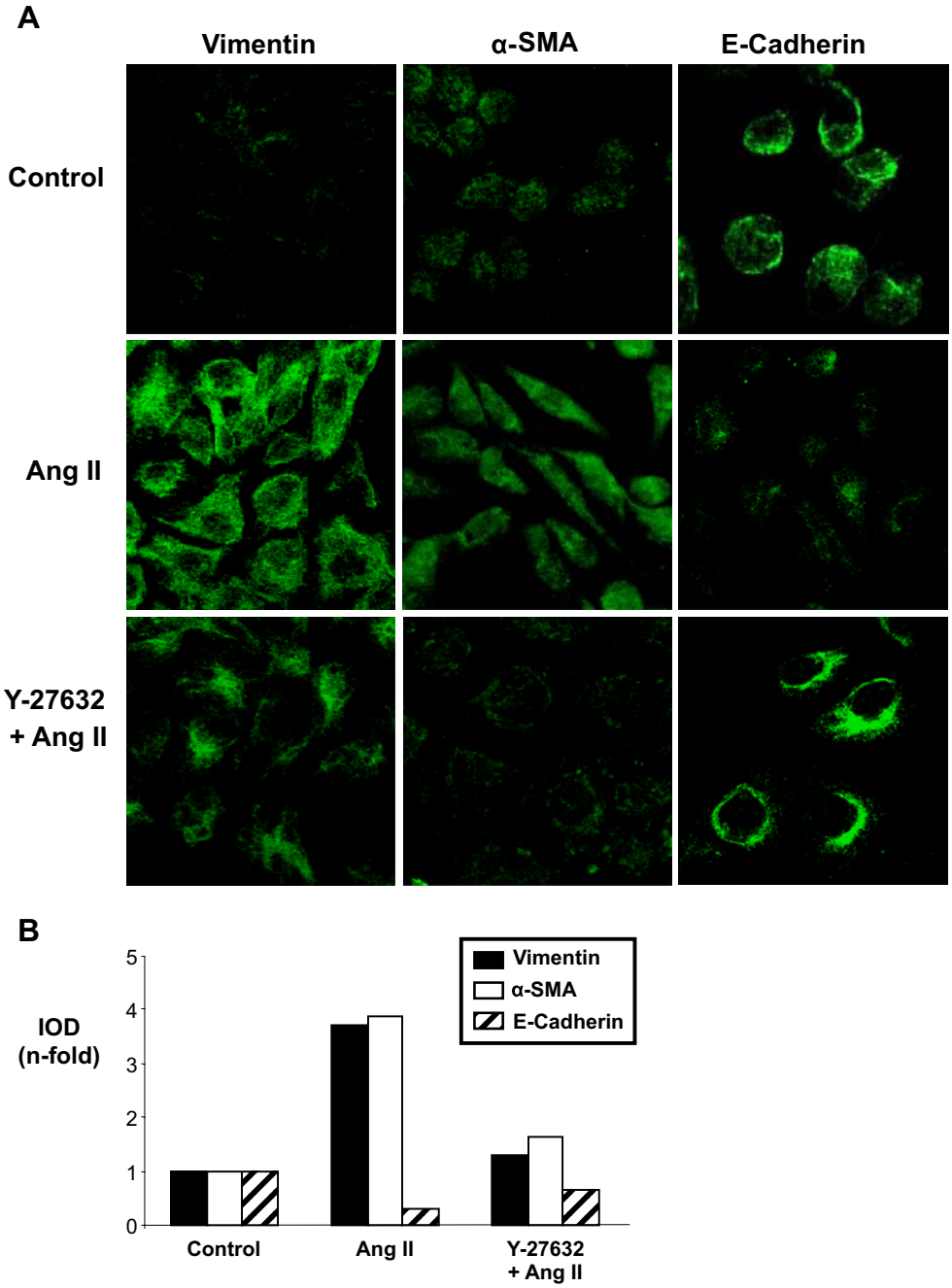
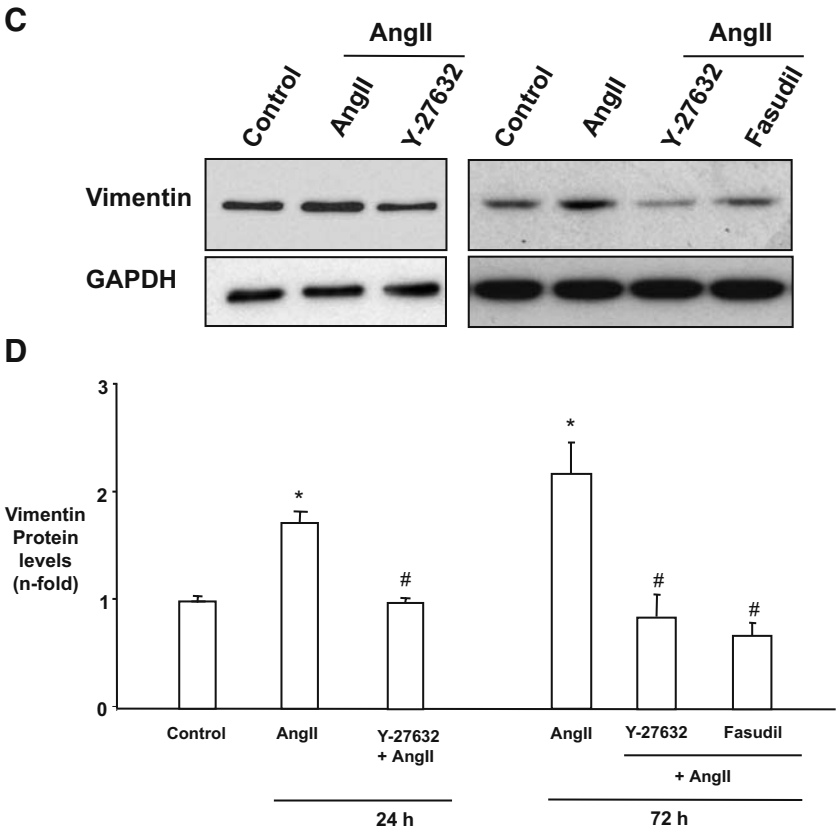


Fig. 3. ROCK inhibitors diminished AngII-induced EMT in human tubuloepithelial cells. Cells were preincubated for 1 h with the ROCK inhibitors Y-27632 or Fasudil (10^{-6} mol/l), and then treated with 10^{-7} mol/l AngII for 24 and 72 h. **A** Vimentin, α-SMA and E-Cadherin were detected by an indirect immunostaining using FICT-labeled secondary antibodies, and evaluated by confocal microscopy. The figures of confocal microscopy show a representative experiment of three done. **B** Quantification of immunofluorescence data expressed as integrated optical density (IOD) as described in "Materials." Vimentin expression was quantified by Western blot after 24 and 72 h of incubation. **C** A representative Western blot and **D** data as mean ± SEM of three independent experiments. *$P<0.05$ *vs* control. #$P<0.05$ *vs* AngII.

Fig. 3. (continued)



positive microfilaments in the cytoplasm of AngII-treated cells, as shown by confocal microscopy in Fig. 1A. Moreover, the loss of E-cadherin induced by AngII was recovered by the three MAPKs inhibitors (Fig. 1A). In Fig. 1B the quantification of immunofluorescence is shown. None of the inhibitors modified EMT markers in control cells (not shown). By Western blot we have further quantified the changes in EMT evaluating vimentin expression levels. AngII caused a rapid induction of vimentin observed at 24 h, which was remained elevated after 3 days. The three MAPKs inhibitors (p38, ERK and JNK) significantly diminished vimentin induction by AngII both at 24 h and 3 days (Fig. 1C and D). These data show the involvement of all three MAPKs in AngII-induced EMT.

The RhoA/ROCK Pathway Participates in AngII-Induced EMT in Human Tubuloepithelial Cells

The involvement of RhoA in EMT caused by AngII was evaluated by transient transfection of several expression vectors. Transient transfection of HK2 cells with a plasmid encoding a dominant negative RhoA isoform (DN–RhoA–GFP) inhibited morphological changes and vimentin induction caused by AngII at 3 days, and had no effect in unstimulated cells. By confocal microscopy, Fig 2A shows unstimulated samples of several cells transfected with DN-RhoA-GFP (green staining). These cells had an epithelial morphology and slight vimentin expression (red staining), presenting similar characteristics than untransfected control cells. In samples stimulated with AngII for 3 days, the non-transfected cells (with negative GFP staining) change their morphology to myofibroblast-like shape and showed a marked vimentin expression. However, DN–RhoA–GFP transfected cells (green staining) remained with a round shape characteristic of epithelial cells. The morphology and staining of all the different cells is more clear in the merge images (Fig. 2A). In cells transfected with the empty vector (GFP staining), stimulation with AngII elicited EMT, as observed in non-transfected cells of the same experiment (Fig. 2B). Moreover, overexpression of a plasmid encoding constitutively active form of RhoA induced a myofibroblast-like phenotype and vimentin expression, confirming that small G protein RhoA participates in EMT (Fig. 2C).

Rho-kinase is a downstrean target of RhoA. Selective pharmacological inhibition of the serine/threonine ROCK I and II, with Y-27632 and fasudil, significantly diminished AngII-induced vimentin immunostaining (Fig. 3A and B) and protein production, both at 24 and 72 h (Western blot; Fig. 3C and D) and restored E-cadherin expression, inhibiting the conversion into myofibroblasts (Fig. 3A and B).

Role of Endogenous CTGF on AngII-Induced EMT

CTGF is a potent profibrotic factor upregulated in renal diseases in association with scarring and sclerosis (21–23). CTGF is a mediator of AngII and TGF-β induced fibrosis and TGF-β-mediated EMT (21,24). We have investigated whether CTGF is a mediator of AngII-induced EMT in HK2

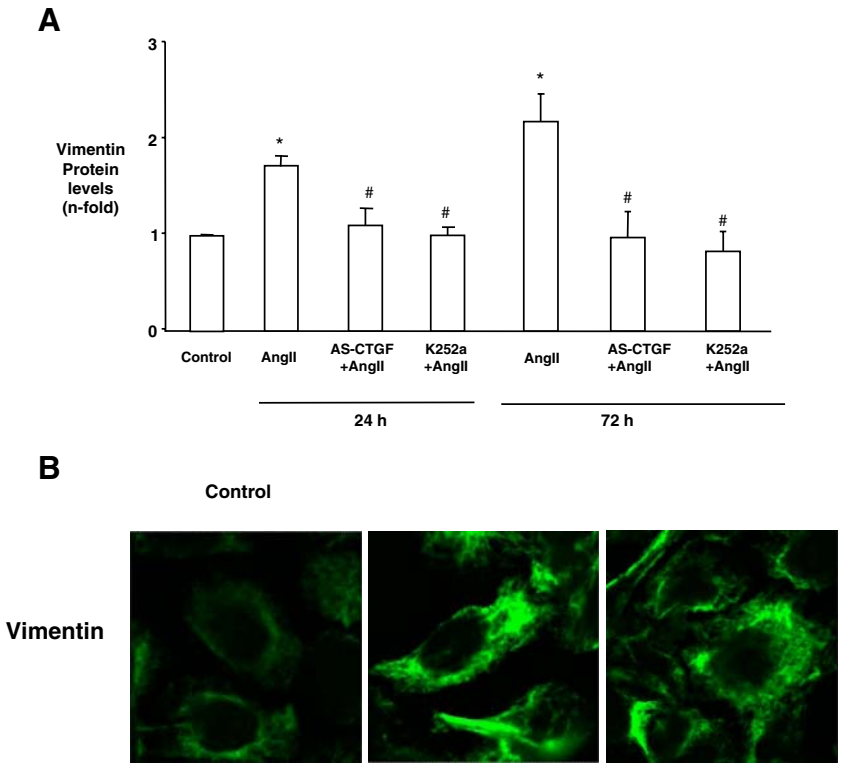


Fig. 4. AngII induces EMT *via* endogenous production of CTGF in human tubuloepithelial cells. CTGF was blocked by antisense oligonucleotide (20 μg/ml) or the CTGF receptor inhibitor K252a (10^{-5} mol/l). Then, cells were stimulated with AngII for 24 and 72 h. **A** Vimentin expression as mean ± SEM of three Western blot experiments. $*P<0.05$ *vs* control. $\#P<0.05$ *vs* AngII. **B** A representative immunocytochemistry experiment of two done. Vimentin was evaluated after 3 days of incubation by an indirect immunostaining using a mouse FICT-labeled secondary antibody.

cells. CTGF was blocked by two methods: an antisense oligodeoxynucleotide, that inhibits its expression (24), and the K252a compound, a receptor tyrosine kinase inhibitor that inhibit internalization and sorting of CTGF receptor (25). The CTGF blockers diminished AngII-induced vimentin expression at 24 h and 3 days (Western blot and confocal microscopy, Fig. 4). These data suggest that CTGF is an early EMT mediator.

Previous studies have demonstrated that AngII upregulates CTGF *via* AT_1 receptors and activation of MAPK, PKC and ROCK pathways (5,26–28). Regarding MAPK cascade, we have found that in human cultured tubuloepitheial cells the three MAPKs inhibitors (p38, ERK and JNK) significantly diminished CTGF overproduction caused by AngII (Fig. 5). In fibroblasts, the inhibitors of ERK1/2 and JNK, but not p38/MAPK, decreased AngII-stimulated CTGF expression (27), while in mesangial cells only the p38 inhibitor SB203580 diminished AngII-induced CTGF production (26), showing a different response depending on the cell type. Pretreatment of HK2 cells with the selective ROCK inhibitor Y-27632 suppressed AngII-induced CTGF protein production (Fig. 5). These results suggest that in human tubuloepithelial cells AngII regulates CTGF *via* activation of three MAPKs (p38, ERK and JNK) and ROCK, showing a similar response to EMT regulation.

Effect of HMG–CoA Reductase Inhibitors on Angiotensin II Induced EMT in Cultured Human Tubuloepithelial Cells

HK2 cells were pretreated for 1 h with two statins: atorvastatin and simvastatin and the effect on EMT caused by AngII was evaluated after 3 days. By confocal microscopy, we have found that both statins, at the dose studied, only partially diminished the phenotypic conversion into myofibroblasts. Fig. 6A shows how several statin-treated cells remain with myofibroblast-like morphology. In these cells the induction of EMT markers (vimentin and α-SMA) and the loss of E-cadherin induced by AngII was only partially recovered by the statins. By Western blot we have observed that atorvastatin partially, but not significantly, diminished vimentin induction caused by AngII at 24 h (Fig. 6C).

As shown in Fig. 4, CTGF is a downstream mediator of AngII-induced EMT. We have recently shown that statins inhibited CTGF production caused by AngII in cultured vascular smooth muscle cells (VSMC) and in Wistar rat aorta (13). In cultured human tubuloepithelial cells atorvastatin did not inhibit CTGF production in AngII-treated cells (Fig. 6D), showing a different regulation between cell types. These data suggest that the partial inhibitory effect of statins on EMT regulation in tubuloepithelial cells could be due to the lack of effect on CTGF regulation.

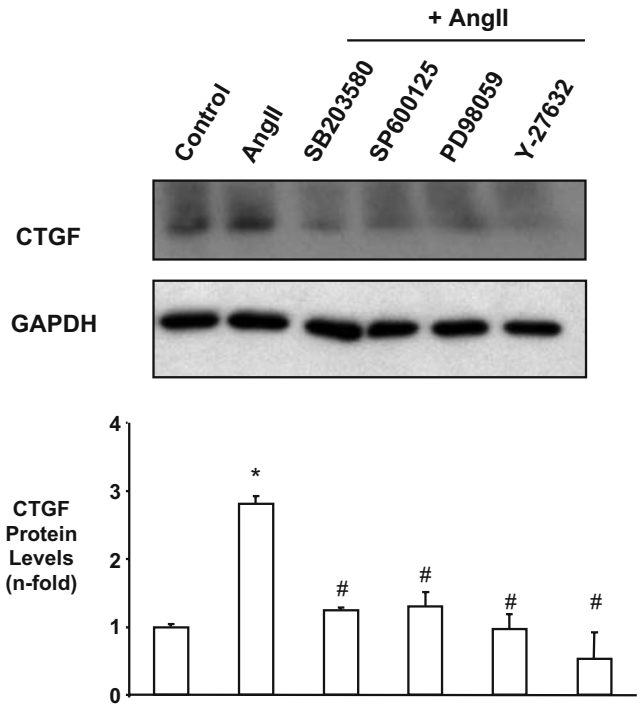


Fig. 5. AngII upregulates production of CTGF *via* MAPK and ROCK activation in human tubuloepithelial cells. Cells were pretreated for 1 h with 10^{-6} mol/l SB203580 (p38 inhibitor), 10^{-5} mol/l of PD98059 (ERK p42/44 inhibitor), SP600125 (JNK inhibitor), 10^{-5} mol/l Y-27632 (ROCK inhibitor), before treatment with 10^{-7} mol/l AngII for 24 h. Figure shows in *top panel* a representative Western blot and in bottom data of total CTGF production as mean ± SEM of four independent experiments. *P<0.05 *vs* control, #P<0.05 *vs* AngII-treated cells.

Atorvastatin Diminishes AngII-Induced MAPK Activation in Cultured Human Tubuloepithelial Cells

In renal cells AngII activates the MAPK pathway [(5,27) and references therein]. In HK2 cells, AngII triggered phosphorylation of all three MAPKs (Fig. 7), with a maximal response between 20 to 30 min. We further investigated whether statins could regulate several AngII-activated intracellular signaling pathways in HK2 cells, as described in VSMC (13). Preincubation with atorvastatin inhibited AngII-induced activation of JNK and ERK1/2 (Fig. 7), showing that in tubuloepithelial cells statins act at cellular level inhibiting AngII responses.

DISCUSSION

The investigation of the molecular mechanisms involved in renal fibrosis could lead to improve current clinical treatments for renal patients. Our *in vitro* data show that specific inhibition of MAPK and ROCK pathways are interesting options for the inhibition of EMT and renal fibrosis.

Several studies have shown that MAPK pathway is involved in EMT and fibrosis. AngII activates MAPK and through this pathway elicits many cellular responses (5,26). In cultured human tubuloepithelial cells we have found that specific inhibitors of all three MAPKs (p38, JNK and ERK1/2) prevented the phenotypic conversion of epithelial cells into myofibroblasts and the loss of E-cadherin observed after 3 days of treatment with AngII, and markedly diminished the induction of the EMT markers vimentin and α-SMA, observed by immunofluorescence and Western blot at 24 h and 3 days (Fig. 8). The MAPK pathway is involved in EMT, fibrosis and cell migration caused by TGF-β (29–31). In different cells, all three MAPKs, p38, ERK and JNK, participates in TGF-β -induced EMT, including in tubuloepithelial cell line NRK52E (32), showing a common intracellular mechanisms for TGF-β and AngII.

Studies done in human renal biopsies from different kidney diseases suggest that MAPK activation in resident and infiltrating cells can be involved in renal damage progression. ERK1/2 activation was associated with cellular proliferation and renal dysfunction (33). In human glomerulonephritis, p38 activation was observed in renal cells and infiltrating cells, correlated with renal dysfunction, proteinuria, inflammatory infiltration and proliferative lesions (34). In experimental models of renal injury activation of JNK have been found in podocytes, endothelial cells, macrophages, T cells and fibroblasts (35,36). In experimental models of renal injury MAPK inhibitors have shown beneficial effects. Treatment with JNK inhibitors reduced renal damage, collagen accumulation and apoptosis in the models of ureteral obstruction and ischemia reperfusion (35–37). Similar data were found in obstructed kidneys of JNK1 and JNK2 deficient mice (38). The pharmacological blockade of ERK1/2 prevented cellular proliferation in experimental glomerulonephritis (39). The effect of p38 inhibitors has been extensively studied. In hypertensive rats specific p38 inhibitors diminished proteinuria, sclerosis and interstitial macrophage migration, *via* suppression of NAD(P)H oxidase and enhanced NO bioavailability, showing end-organ protection regardless of overt antihypertensive action (40,41). In high-renin homozygous transgenic rats, p38 inhibition reduced both glomerular and tubulo-interstitial fibrosis and induction of α-SMA expression (42,43). In double transgenic rats for renin and angiotensinogen, p38 inhibition diminished renal expression of CTGF, TNF-α, IL-6, macrophages infiltration and fibrosis (44). Recently, it has been developed a novel strategy that inhibit p38 within proximal tubular cells, by using a renal-specific conjugate of the p38 inhibitor SB202190 and the carrier lysozyme. In the model of ischemia-reperfusion in rats this compound reduced intrarenal p38 phosphorylation and α-SMA protein expression (45). These data suggest that pharmacological inhibition of MAPK pathway could be an important therapeutic approach for renal diseases.

RhoA participates in some AngII responses, including vasoconstriction, premyofibril formation and cell hypertrophy (4,46). Several findings suggest that RhoA/ROCK pathway is implicated in the etiology of renal fibrosis. In our *in vitro* experiments, the transient transfection of a RhoA dominant negative vector or the use of two ROCK inhibitors (Y-27632 and Fasudil) clearly demonstrated that RhoA/ROCK pathway regulates AngII-mediated EMT (Fig. 8). The small G protein RhoA participates in TGF-β-mediated EMT (47,48). The mechanisms of this process involve RhoA degradation by recruitment of ubiquitin ligase Smurf1 (49). In experimental models of renal damage, such as unilateral ureteral obstruction, nephrectomized spontaneously hypertensive rats, L-NAME-treated and AngII infusion, ROCK inhibition improved glomerular and tubulointerstitial injury scores and fibrosis. In some of these models ROCK inhibition diminished gene

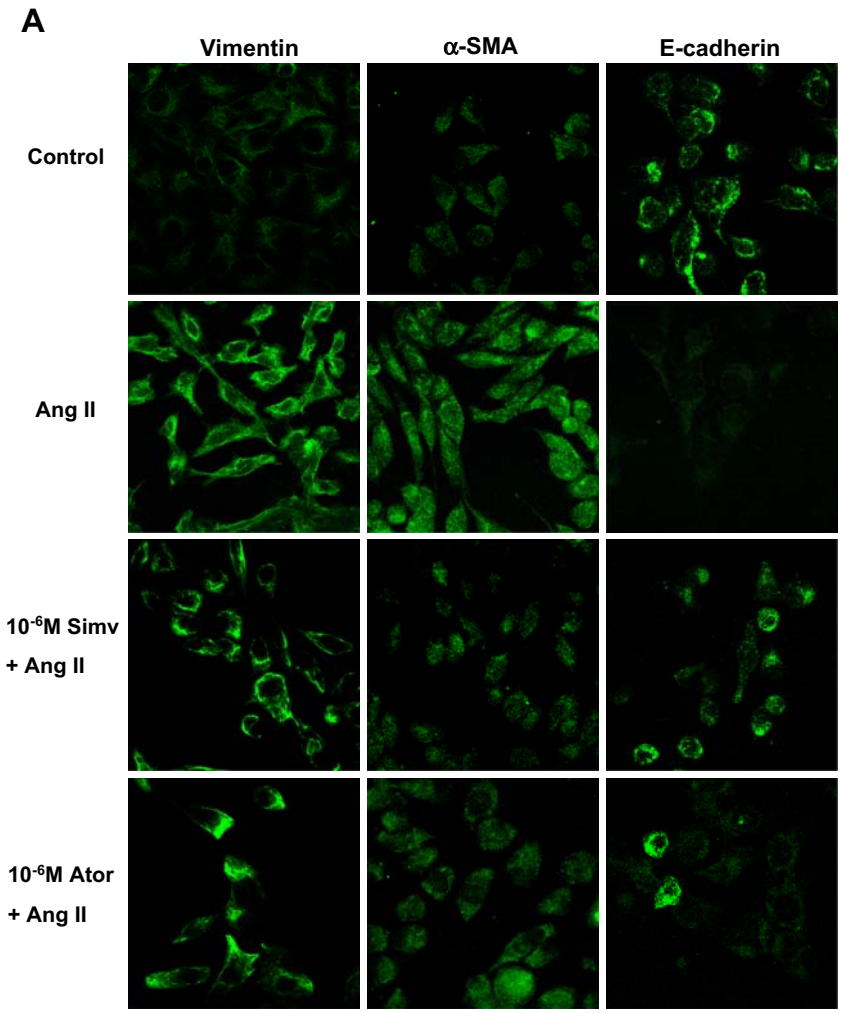
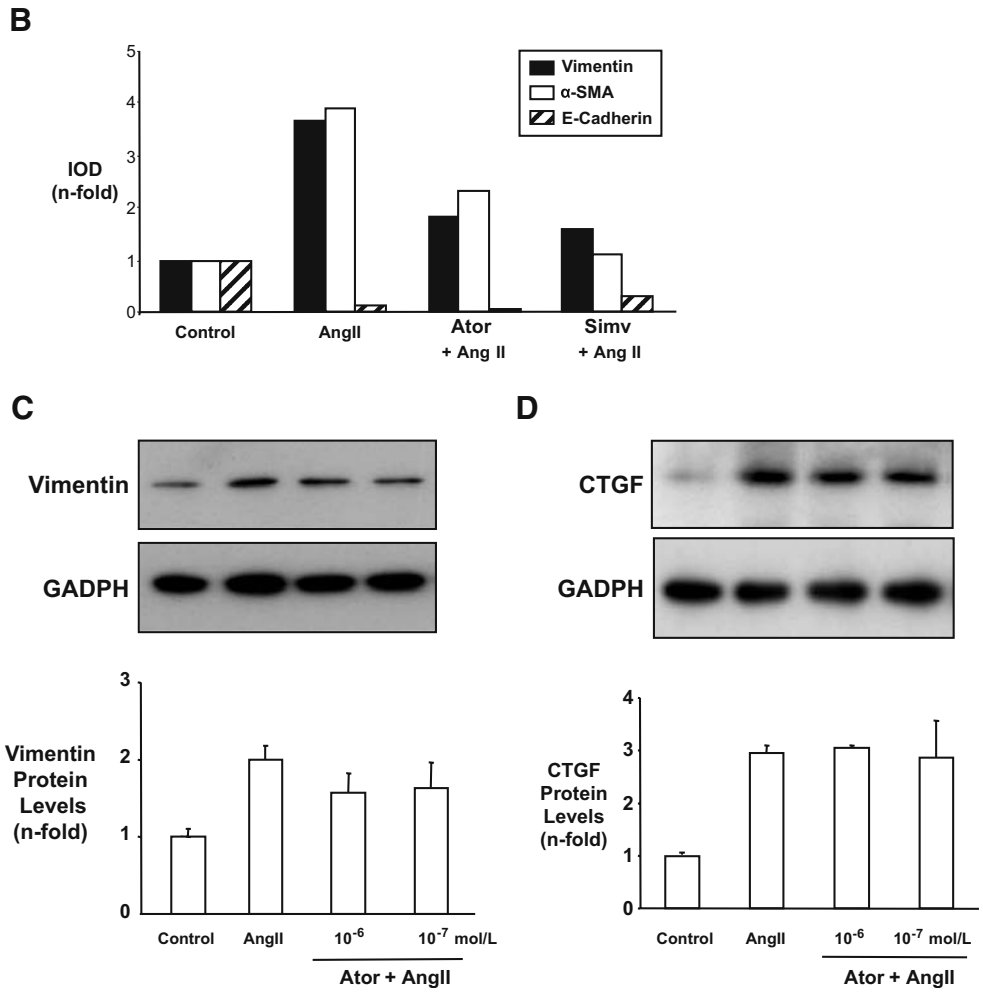


Fig. 6. Effect of HMG-CoA reductase inhibitors on AngII-induced EMT. Cells were pretreated for 1 h with two statins: atorvastatin or simvastatin (10^{-6}mol/l) and then stimulated with 10^{-7} mol/l AngII for 3 days. **A** Vimentin, α-SMA and E-cadherin were detected by an indirect immunostaining using a mouse FICT-labeled secondary antibody, and evaluated by confocal microscopy. The figures of confocal microscopy show a representative experiment of three done. **B** Quantification of immunofluorescence data expressed as integrated optical density (IOD) as described in "Materials and Methods." **C** HK2 cells were pretreated for 1 h with atorvastatin (10^{-6}–10^{-7} mol/l) and then stimulated with 10^{-7} mol/l AngII for 24 h and vimentin expression was quantified by Western blot. Data are expressed as mean ± SEM of three experiments. **P*<0.05 *vs* control, #*P*<0.05 *vs* AngII. **D** HMG–CoA reductase inhibitors did not modulate CTGF production caused by AngII. HK2 cells were pretreated for 1 h with atorvastatin (10^{-6} and 10^{-7}mol/l) and then stimulated with 10^{-7}mol/l AngII for 24 h. Results of total CTGF production were obtained from densitometric analysis and expressed as ratio CTGF/GAPDH as *n*-fold over control. Figures show in top panel a representative Western blot and bottom data total CTGF production as mean ± SEM of three independent experiments. **P*<0.05 *vs* control.

overexpression of α-SMA, TGF-β, CTGF and matrix proteins (50,51). Recently, specific tubular inhibition of ROCK has shown renal protective effects in ischemia-reperfusion in rats (52). These investigations support the idea that treatments that inhibit RhoA/ROCK pathway in tubuloepithelial cells could be an appropriate choice as therapeutic strategies in chronic renal diseases.

Many efforts have been done to find a biomarker for the progression of chronic renal diseases, but until now there is not a good candidate. CTGF is upregulated in many human renal diseases and mediates TGF-β-induced fibrosis and EMT (21,53). We have previously shown that CTGF is a downstream mediator of AngII-induced renal fibrosis (24). In this paper, we have observed that CTGF blockade by a CTGF antisense oligonucleotide or an inhibitor of CTGF-receptor, diminished vimentin induction caused by AngII both at 24 and 72 h, showing that CTGF also contributes to AngII-induced EMT. Similar findings were previously described (18), supporting the importance of CTGF as a mediator of EMT. Moreover, recently it have been demonstrated that the treatment with a CTGF antisense oligonucleotide ameliorates renal damage in experimental diabetes (54).

Fig. 6. (continued)



In these animals a correlation between renal and urine levels of CTGF has been found, indicating the potential importance of this growth factor as a biomarker in renal diseases. In different cell types MAPKs and ROCK are involved in CTGF regulation. In fibroblasts, ERK1/2 and JNK, but not p38 inhibition, decreased AngII-induced CTGF upregulation (28), while in mesangial cells only the p38 inhibitor SB203580 diminished CTGF (27). In cultured renal fibroblasts activation of Rho is involved in CTGF overexpression caused by TGF-β and AngII (55,56). The involvement of Rho in CTGF regulation has been described in many cell types, including VSMC and lung fibroblasts (13,57). In this paper we have observed that in human tubuloepithelial cells AngII regulates CTGF *via* activation of three MAPKs (p38, ERK and JNK) and ROCK, showing a similar response to EMT regulation (Fig. 8). Although more studies are needed to define whether CTGF can be used as a biomarker in renal patients, the effect of these kinase inhibitors support the idea that CTGF could be a molecular target for the regulation of EMT.

The HMG-CoA reductase inhibitors are effective in controlling hypercholesterolemia, even in advanced stages of renal failure and in patients who are on chronic dialysis, and present cardiovascular protective effects (58), however their renoprotective effects in human renal diseases are not proven. Although several experimental models of kidney injury have shown beneficial effects, there are some contradictory data. A meta-analysis of several smaller studies of patients with various forms of renal diseases concluded that lipid-lowering drugs can reduce the decline of the glomerular filtration rate, but large clinical trials are warranted (59). Chronic treatment with the hydrophilic rosuvastatin, but not the lipophilic simvastatin had renoprotective effects in spontaneously hypertensive stroke-prone rats, a model characterized by proteinuria, inflammatory cell infiltration, α-SMA-positive cells, degenerative changes in podocytes, and severe fibrosis (15). In murine adriamycin nephropathy statins failed to ameliorate renal damage (60). In the model of unilateral ureteral obstruction in rats simvastatin diminished renal interstitial inflammation and fibrosis. Simvastatin also prevented tubular activation and transdifferentiation, as shown by decreased vimentin and α-SMA expression (61). In cultured human tubuloepithelial cells we have found that two statins, atorvastatin and simvastatin only partially diminished AngII-induced EMT changes. Similar findings were found with pravastatin in response to TGF-β mediated EMT and extracellular matrix deposition, and only the combination with PPAR-gamma agonists markedly inhibited these processes (17). The pleiotropic effect of statins are due to their inhibition of cellular responses, as a result of the inhibition of the mevalonate pathway induced by these agents, which

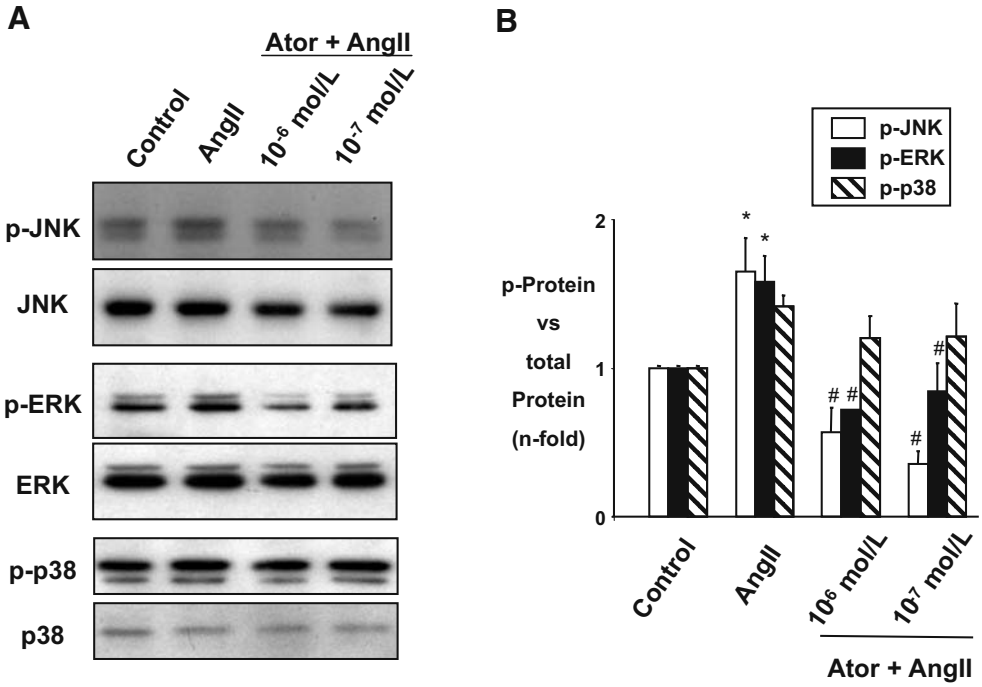


Fig. 7. Atorvastatin diminishes MAPK activation in AngII-treated human tubuloepithelial cells. Cells were pretreated for 1 h with atorvastatin (ator: 10^{-6}–10^{-7} mol/l), and then stimulated with 10^{-7} mol/l AngII for 20 (for JNK) and 30 min (ERK and p38). Figure shows a representative Western blot of phospho-JNK (p-JNK1/2), phospho-ERK (p-ERK1/2) and phospho-p38 (p-p38), JNK, ERK and p38 (used as controls). Figure show data as mean ± SEM of three to four experiments. *$P<0.05$ *vs* control. #$P<0.05$ *vs* AngII.

includes the activation of the small G protein Rho and MAPKs (11–13). In tubuloepithelial cells we have observed that atorvastatin inhibited AngII-induced MAPKs activation. Similar findings were observed with lovastatin, simvastatin, and pravastatin in the reduction of RhoA and Rac1 activation and in the inhibition of EMT caused by activated peripheral blood mononuclear cells conditioned-medium (16). However, our studies in cultured tubuloepithelial cells showed that the inhibitory effect of statins was lower than those of kinase inhibitors on AngII-induced EMT. Interestingly, we have observed that atorvastatin did not diminish CTGF production caused by AngII, showing a different response to MAPKs and ROCK inhibitors, which abolished AngII-induced CTGF upregulation. The regulation of CTGF seems to be dependent on the cell type and stimuli, as described above for MAPK pathway. In this sense, in Wistar rat VSMC and several fibroblasts cell lines statins diminished CTGF induced by AngII and TGF-β (13,62–64), while this effect was not observed in tubuloepithelial cells. Future studies are needed to further investigate the mechanisms involved in statins action in the kidney and in renal cells.

CONCLUSION

Our data show that MAPKs and ROCK inhibitors abolished CTGF upregulation and EMT, suggesting that the blockade of these pathways could be an important therapeutic choice for renal diseases. Future *in vivo* investigation of the effect of kinase inhibitors in chronic renal diseases could improve the current clinical treatments of renal patients.

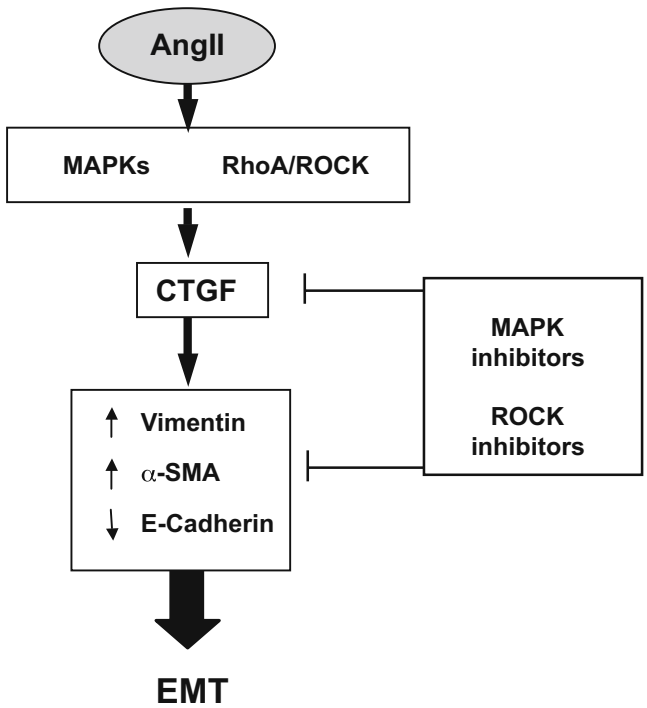


Fig. 8. AngII through the activation of the MAPKs cascade and the RhoA/ROCK pathway regulates CTGF and EMT markers.

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3. Gremlin se induce en patologías renales progresivas, asociado con fibrosis y activación de TGF-β/Smad.

Nuestro siguiente objetivo ha sido evaluar si Gremlin puede ser considerado como un nuevo mediador de fibrosis renal y TEM, evaluando la posible implicación de la ruta TGF-β/Smad.

Estudios previos *in vivo* e *in vitro* sugieren que Gremlin, antagonista de BMP-7, podría tener un rol clave en la patogénesis de la nefropatía diabética como mediador de las acciones de TGF-β. [27,87,68,100,126] En muestras de biopsias de pacientes con distintas nefropatías se ha evaluado la expresión génica y proteica de Gremlin, y su posible correlación con TEM y fibrosis renal. Además, en algunos casos se ha determinado si existe co-localización con sobreexpresión de TGF-β o activación de la ruta Smad. Se han realizado varios estudios, cuyos resultados se muestran en las 3 publicaciones siguientes, pero se comentan todos en conjunto al solapar en objetivos y conclusiones finales.

En el primer trabajo se estudiaron 30 biopsias renales de pacientes con glomerulonefritis pauci-inmune. Observamos marcada expresión de Gremlin, ARNm y proteína, en co-localización con TGF-β, en las células epiteliales parietales y monocitos presentes en las crescientes celulares y fibrocelulares de las biopsias estudiadas. También se observó importante expresión de Gremlin en células túbulo-epiteliales e intersticiales infiltrantes, lo que se correlacionó con el grado de fibrosis túbulo-intersticial. En las células túbulo-epiteliales que expresaron TGF-β y Gremlin observamos además activación de Smad. Por lo tanto, postulamos que Gremlin tendría un rol en el proceso de fibrosis secundario a glomerulonefritis pauci-inmune tanto en células glomerulares como tubulares. La co-localización entre Gremlin y TGF-β sugiere que Gremlin podría ser un mediador importante de las acciones de TGF-β.

En el segundo trabajo, se ha evaluado la expresión renal de Gremlin en la nefropatía crónica del injerto (NCI). Esta patología es la principal causa de disfunción crónica y pérdida tardía del injerto. El proceso de TEM sería responsable de la presencia de miofibroblastos activados en el intersticio y la ruta TGF-β/Smad sería la principal vía de señalización implicada en el proceso. En este estudio se han evaluado 33 biopsias renales, 16 de las cuales presentaban NCI y las otras 17 correspondían a controles. En las biopsias de pacientes trasplantados con NCI detectamos sobre-expresión de TGF-β, en co-localización Gremlin ARNm y proteína, principalmente en áreas de fibrosis túbulo-intersticial. En los mismos túbulos encontramos disminución en la expresión de E-cadherina e inducción en la expresión de Vimentina y α-SMA. Nuestros datos sugieren que Gremlin podria ser un mediador de las acciones de TGF-β y tener un rol en la TEM observada en la NCI.

El tercer trabajo recoge una gran muestra de 125 biopsias de diferentes glomerulopatías progresivas y no progresivas, en las cuales se evaluó la expresión de Gremlin ARNm y proteína. Encontramos una marcada expresión de Gremlin en todas las nefropatías estudiadas, excepto en la enfermedad por cambios mínimos, que es una nefropatía no progresiva. En el tejido renal normal, tampoco se ha descrito expresión de Gremlin.[27] Gremlin se encontró principalmente en las células túbulo-epiteliales e intersticiales infiltrantes. El grado de fibrosis túbulo-intersticial se correlacionó con la expresión de Gremlin ARNm. En cortes seriados de nefropatías progresivas observamos disminución en la expresión de E-cadherina y aumento de los marcadores mesenquimales en co-localización con la expresión de Gremlin. Además, en las células de túbulo-epiteliales se encontró co-expresión de ARNm de Gremlin y TGF-β, y activación de Smad.

Todo lo anterior apoya la afirmación de que Gremlin es un factor patogénico común en glomerulopatías humanas progresivas, cuya expresión renal se correlaciona con el grado de fibrosis túbulo-intersticial. Además, estos estudios sugieren que Gremlin podría actuar modulando el proceso de TEM en el riñón, mediante la regulación de la ruta TGF-β/Smad.

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Original Article

Expression of gremlin, a bone morphogenetic protein antagonist, in glomerular crescents of pauci-immune glomerulonephritis

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Abstract

Background. Recent evidence *in vitro* and *in vivo* suggests that gremlin, a bone morphogenetic protein antagonist, is participating in tubular epithelial mesenchymal transition (EMT) in diabetic nephropathy as a downstream mediator of TGF-β. Since EMT also occurs in parietal epithelial glomerular cells (PECs) leading to crescent formation, we hypothesized that gremlin could participate in this process. With this aim we studied its expression in 30 renal biopsies of patients with pauci-immune crescentic nephritis.

Methods. Gremlin was detected by *in situ* hybridization (ISH) and immunohistochemistry (IMH) and TGF-β by ISH and Smads by southwestern histochemistry (SWH). Phosphorylated Smad2, CTGF, BMP-7, PCNA, α-SMA, synaptopodin, CD-68, and phenotypic markers of PECs (cytokeratin, E-cadherin), were detected by IMH. In cultured human monocytes, gremlin and CTGF induction by TGF-β was studied by western blot.

Results. We observed strong expression of gremlin mRNA and protein in cellular and fibrocellular crescents corresponding to proliferating PECs and monocytes, in co-localization with TGF-β. A marked over-expression of gremlin was also observed in tubular and infiltrating interstitial cells, correlating with tubulointerstitial fibrosis ($r = 0.59$; $P < 0.01$). A nuclear Smad activation in the same tubular cells, that are expressing TGF-β and gremlin, was detected. In human cultured monocytes, TGF-β induced gremlin production while CTGF expression was not detected.

Conclusion. We postulate that gremlin may play a role in the fibrous process in crescentic nephritis, both in glomerular crescentic and tubular epithelial cells. The co-localization of gremlin and TGF-β expression found in glomeruli and tubular cells suggest that gremlin may be important in mediating some of the pathological effects of TGF-β.

Keywords: BMP-7 antagonist; crescentic glomerulonephritis; epithelial mesenchymal transition (EMT); gremlin; TGF-β; Smad

Introduction

Gremlin, a member of the cysteine knot protein super family 1, is a highly conserved glycosylated and phosphorylated secreted protein present both on the external cell surface and within the ER-Golgi compartment of a variety of cell types [1]. Gremlin, as bone morphogenetic protein (BMP) antagonist, has been reported to influence diverse processes in growth, differentiation and development, in many cases by heterodimerization with BMP-2, -4 and -7, thereby inhibiting the ability of these ligands to bind to their receptors. Recent work has established that gremlin mediates its action via induction of epithelial to mesenchymal feedback signalling. Metanephric renal (and limb bud) organogenesis occurs via BMP antagonism and thus gremlin is confirmed as the essential extracellular signal which initiates renal development [2].

The induction of gremlin in cultured human mesangial cells exposed to high glucose and transforming growth factor-β (TGF-β) *in vitro* and in kidneys from diabetic rats *in vivo*, has been reported [3]. Recently we have shown that gremlin mRNA and protein are highly expressed in the tubular compartment of advanced human diabetic nephropathy [4]; kidney biopsies from patients with diabetic nephropathy had significantly increased gremlin expression when compared with normal kidney or biopsies from patients with non-scarring renal disorders such as minimal change disease. Gremlin expression was most pronounced in areas of interstitial fibrosis and it is co-localized with TGF-β [4].

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Since gremlin expression has been induced by TGF-β1 in renal proximal tubular cells *in vitro* undergoing transdifferentiation to a fibroblast phenotype, a role of gremlin in the epithelial mesenchymal transition (EMT) process, as BMP-7 antagonist has been proposed [2,3].

On the other hand, the discovery of TGF-β/Smad-signalling pathway has allowed to study the intracellular mechanisms of TGF-β and BMP-7 at the EMT [5]. TGF-β signalling is mediated by nuclear translocation of phosphorylated Smad 2 and Smad 3 and BMP-7 by Smad 1, Smad 5 and Smad 8 [6].

Recently, Bowman's epithelial-mesenchymal transdifferentiation, identified by alpha-smooth muscle actin (α-SMA) expression in the evolution of glomerular crescent formation, has been reported [7–9]; so far we hypothesize a role for gremlin in the scar formation of crescentic glomerulonephritis. The purpose of this study is to examine the presence of gremlin in the glomerular crescents formation in 30 renal biopsies from patients with pauci-immune crescentic glomerulonephritis and correlate its expression with TGF-β and markers of EMT, trying to define whether gremlin is a phenotypic modulator in the formation of glomerular crescents.

Materials and methods

Assessment of the pattern of gremlin expression by in situ hybridization (ISH)

Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation at the Division of Nephrology, Austral University, Valdivia, Chile. The samples were studied after obtaining patient consent and the project was approved by the local hospital ethics committee. Renal biopsies from 30 patients with pauci-immune crescentic nephritis before specific treatment were studied.

Control human kidney specimens ($n = 5$) were taken from normal portions of renal tissue from patients who underwent surgery because of localized renal tumours.

ISH was performed as described previously for antisense TGF-β probe (R&D Systems, Minneapolis, MN, USA) [10], and with the following modifications for biotin-labelled human gremlin probes (Invitrogen, Carlsbad, CA, USA). The gremlin probes were as follows: 478 antisense 5′-TGAAAGGAACCTTCCTCCTTCC-3′,2416 antisense 5′-ATGGGAGAGCACTGGATCAAAA-3′ and 3553 antisense 5′-CAGGCACTGACTCAGGAAGACA-3′ For gremlin analysis, pretreatment with endogenous biotin blocking system (Dako Co, Carpinteria, CA, USA) was performed prior to proteinase K digestion. The sections were incubated with a pre-hybridization solution (Dako, mRNA ISH Solution) for 60 min at 37°C and with the antisense probe overnight at 37°C. The slides for gremlin were washed with 2 × SSC and 1 × SSC for 10 min at room temperature and then with 0.5 × SCC for 20 min at 37°C.

Detection was performed with avidin-alkaline phosphatase conjugate (Dako) for 30 min at room temperature, washed 5 min with 1 × TBS and using NBT-BCIP as the enzyme substrate for 120 min at 37°C (R&D Systems). Tissues were then dehydrated in ethanol series and mounted in Canadian balsam (Polysciences Inc., Warrington, PA, USA).

The specificity of the reaction was confirmed: (i) by demonstrating the disappearance of hybridization signal when RNAse (100 μg/ml) (Sigma Chemicals Co., St. Louis, MO, USA) was added in 0.05 M Tris after the digestion with proteinase K; (ii) by the use of a sense probe (R&D Systems); (iii) with a negative control (Plasmid DNA) (Dako) and (iv) without probe.

For gremlin ISH slides, Dako nuclear fast red was used for 10 min.

Immunohistochemistry (IMH)

For light microscopy, kidney tissues were fixed in 4% buffered formalin, or Bouin, dehydrated and embedded in paraffin by conventional techniques. Sections were stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS), and silver methenamine. Paraffin embedded biopsy specimens were used for detection of gremlin, BMP-7, CTGF, pSmad2, α-SMA, PCNA, e-cadherin, cytokeratin, synaptopodin, and macrophage marker (CD68).

The following primary antibodies were employed: rabbit polyclonal anti-gremlin (ABGENT, AP6133a, San Diego CA, USA); goat polyclonal anti-human BMP-7 (Santa Cruz Biotechnology, CA, USA); rabbit polyclonal anti-human CTGF (ABCAM, ab6992, Cambridge, UK); rabbit polyclonal anti-Smad2 (phospho S465) ABCAM ab5490; mouse anti-PCNA clone PC10 (Dako); mouse anti-human alpha-smooth muscle actin clone 1A4 (Dako); mouse anti-human e-cadherin clone 36B5 (Novocastra, Newcastle, UK); mouse anti-multi-cytokeratin NCL-AE1/AE3 clone AE1 y AE3 (Novocastra); mouse anti-human synaptopodin clone G1D4 (Progen, Heidelberg, Germany); mouse anti-CD68 (Dako).

Briefly, 5 μm thick renal sections, Bouin- or formalin-fixed were deparaffinized through xylene, alcohol and distilled water. Endogenous peroxidase was blocked by 3% H_2O_2 for 15 min and then the sections were treated in a microwave oven in a solution of 0.1 mM citrate buffer pH 6.0 for 10 min or EDTA buffer 1 mM pH 8.0 for synaptopodin detection. After blocking, the sections were incubated overnight at 4°C with the specific primary antibody. The sections were then incubated with the correspondent biotinylated secondary antibodies for 30 min at 22°C. After three rinses in Tris saline buffer, they were incubated with streptavidin-peroxidase (Dako) 1/1000 for 30 min. Color was developed with substrate (Dako) and then counterstained with haematoxylin, dehydrated, and mounted with Canadian balsam (Polysciences, Inc.). The specificity was checked by omission of primary antibodies and use of non-immune sera.

Southwestern histochemistry (SWH)

This technique has been described by Isono *et al.* [11] and recently reported in our laboratory for NF-κB detection [12].

Briefly, complementary oligonucleotides containing a Smad binding consensus sequence (21) were synthesized by Invitrogen as follows: 5′-GAGTATGTCTAGACTGACAA TGTAC-3′. After annealing with their complementary DNA (80°C during 2 min), the probe was labelled with digoxigenin (DIG oligonucleotide three-end labelling kit),

(Boehringer Mannheim, Mannheim, Germany). Paraffin-embedded kidney sections were dewaxed, rehydrated and incubated with 5 mM levamisole (Sigma Chemical Co.) to inhibit endogenous alkaline phosphatase, and fixed with 0.2% p-formaldehyde for 30 min at 28°C. Sections were subsequently digested with pepsin A (433 U/mg; Sigma), washed twice with buffer 1 (10 mM HEPES, 40 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 0.25% BSA, pH 7.4), and then with 0.1 mg/ml DNAse I, washed once with buffer 2 (10 mM HEPES, 40 mM NaCl, 1 mM DTT, 10 mM EDTA, 0.25% BSA, pH 7.4) to stop the reaction. The labelled probe (100 pM) diluted in buffer 1 containing 0.5 mg/ml poly (dI-dC) (Pharmacia LKB, Piscataway, NJ, USA) was applied overnight at 37°C.

After washing, sections were incubated for 1 h in blocking solution (0.01 × SSC, 0.01% SDS, 0.03% Tween 20, 0.1 M maleic acid, 0.15 M NaCl, pH 7.5), and with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:250 in blocking solution; Boehringer Mannheim) overnight at 4°C. The color reaction was developed using (NBT/BCIP) (Dako).

Immunohistochemistry quantification

The surface area labelled was evaluated by quantitative image analysis using a KS 300 imaging system 3.0 (Zeiss, München-Hallbergmoos, Germany).

For each sample, the mean staining area was obtained by analysis of 20 different fields (20×). Quantification was done twice, independently, and interassay variations were not significant. The staining score is expressed as percentage/mm^2.

Tubulointerstitial cell infiltration and interstitial fibrosis was classified into four groups according to the extent of them and the presence of tubular atrophy and degeneration: (i) normal, (ii) involvement up to 25% of the cortex, (iii) involvement of 26 to 50% of cortex, and (iv) extensive damage involving more than 50% of the cortex.

In vitro studies. Human monocyte cells (THPs cell line) were used. Cells were grown in RPMI with 10% FCS. Cells were serum starved for 24 h before the experiments. Cell culture reagents were from Life Technologies, Inc. Recombinant TGF-β was from Preprotech. Protein levels were evaluated by western blot. Cell samples were homogenized in lysis buffer and protein content was determined by the BCA method (Pierce, Rockford, IL, USA). Proteins (20 µg/lane) were separated on 15% SDS-PAGE gels, transferred, blocked and incubated with Gremlin antibody overnight at 4°C. Detection was made with corresponding peroxidase-conjugated secondary antibody and developed using an ECL chemiluminiscence kit (Amersham). The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad, Spain). Results are expressed as n-fold increase over control as mean ± SEM of experiments made.

Statistical analysis

The statistical analysis was performed with the GraphPad Instat, GraphPad Software, San Diego, CA, USA. The results of the clinical data are expressed as the mean ± SD. A Spearman correlation was used to correlate tubule interstitial gremlin expression and tubule interstitial cell infiltration and fibrosis. A Pearson correlation was used to correlate the gremlin mRNA and protein expression.

Results

The clinical data of the patients studied are presented in Table 1. Biopsies from 30 patients with pauci-immune crescentic glomerulonephritis, 80% of them with rapidly progressive GN (mean s. creat. 7.8 ± 4.5 mg/dl), and with different range of proteinuria, were included in these studies. The mean age of the patients was 47 ± 20 years old (median 50) and 18 were females. The percentage of glomerular crescents was over 50%, the immunofluorescence microscopy was negative or with rare immune deposits in all the cases (pauci-immune); 43% of the patients were P-ANCA positive and 20% were C-ANCA positive.

The cellular components of these crescents are mainly parietal epithelial cells, cytokeratin-positive (Figure 1B) and dysregulated parietal epithelial cells negative for cytokeratin, with different participation of infiltrating monocyte/macrophage cells (CD68-positive cells) (Figure 1C). All these crescentic cells are mainly PCNA-positive cells as it is shown in Figure 1A. PCNA was also expressed in numerous tubular and interstitial cells. On the other hand, these crescentic cells, are negative for synaptopodin (Figure 1F), a phenotypic marker of podocytes, and negative for E-cadherin (Figure 1E), a tubular epithelial marker, that is lost during the glomerular epithelial-myofibroblast transition process. In glomerular crescents in which a fibrous transition to fibrocellular or fibrous crescent is occurring, the cellular components are replaced by proliferating α-SMA (+) myofibroblasts, within a collagen-rich sclerotic crescent (Figure 1D).

The gremlin expression was studied by *in situ* hybridization and by immunohistochemistry, as it is illustrated in Figure 2. Gremlin was not expressed in normal human kidney (Figure 2C). Conversely, abundant gremlin mRNA expression (Figure 2A–D) and gremlin protein staining (Figure 2B) were observed in cellular glomerular crescents. As it is shown in Figure 2B, the gremlin protein was detected in proliferating cellular crescents, and this expression was not observed in those fibrous crescents. Unexpectedly, abundant gremlin expression was also observed in tubular epithelial cells, as it is illustrated in Figure 2A, and a strong correlation between the gremlin mRNA and protein expression was observed in the samples studied ($r = 0.8$; $P < 0.01$).

Moreover, gremlin expression was most prominent in areas of tubulointerstitial fibrosis, and was also observed in interstitial inflammatory cells, as it is illustrated in Figure 2E, with a strong correlation between the tubular and interstitial gremlin expression and the tubulointerstitial fibrosis score ($r = 0.59$; $P < 0.01$) (Figure 5).

Since gremlin induction has been reported in tubular epithelial cells exposed to TGF-β, we studied the

Table 1. Clinical and biological data from the 30 patients with pauci-immune crescentic glomerulonephritis

Patient	Age (years)/ Gender	Initial S_{Cr} (mg/dl)	Urinary findings	Proteinuria (g/24 h)	Clinical features	ANCA	Follow-up
1	57/F	16.7	P: 75 mg/dl; RBC: 50–62	N.A.	RPGN Pulmonary haemorrhage	c-ANCA	On chronic dialysis
2	50/F	8.2	P: (–); RBC: 5–10	N.A.	RPGN	c-ANCA	After 3 years, stable serum cretainine (3 mg/dl)
3	22/M	8.8	P: 500 m/dl,RBC: 6–8; hyaline, granular, RBC casts; oval fat bodies	3.7	RPGN Uraemic syndrome	(–)	Transplanted, after 6 years on chronic dialysis
4	73/F	4.9	P: 100 mg/dl; RBC: >100	N.A.	RPGN	p-ANCA	Death on chronic dialysis
5	66/F	5.7	P: 25 mg/dl; RBC: 30–40; WBC: 6–8	0.42	RPGN Pulmonary haemorrhage	c-ANCA	Death on chronic dialysis (CNS vasculitis)
6	72/M	8.2	P: 75 mg/dl; RBC: >100	N.A.	RPGN	c-ANCA	After 5 years, stable serum creatinine (1.4 mg/dl)
7	47/M	7.8	N.A.	N.A.	RPGN	(–)	Unknown
8	50/F	7.7	P: (–); RBC: 6–8	N.A.	RPGN	c-ANCA	On chronic dialysis
9	22/F	10.0	P: 500 mg/dl; RBC: >100; granular, waxy, broad, hyaline, fatty, RBC casts	2.4	RPGN Pulmonary haemorrhage	p-ANCA	Transplanted, after 2 year on chronic dialysis
10	58/M	8.0	P: 100 mg/dl; RBC: >100; granular, hyaline, fatty, RBC casts	6.4	RPGN	(–)	Death on chronic dialysis
11	21/M	12.0	P: 300 mg/dl; RBC: 180–200; WBC: 8–20	N.A.	RPGN	(–)	After 10 years, stable serum creatinine (1.9 mg/dl)
12	34/M	6.3	P: 500 mg/dl; RBC: >100	8.1	RPGN	(–)	On chronic dialysis
13	62/F	4.9	P: 75 mg/dl; RBC: 0–4	N.A.	RPGN	c-ANCA	After 5 years, stable serum creatinine (1.6 mg/dl)
14	41/M	2.9	P: 150 mg/dl; RBC: >100; granular, epithelial, waxy, RBC casts; oval fat bodies	5.6	Nephrotic syndrome, pulmonary haemorrhage	p-ANCA	Transplanted, after chronic dialysis
15	10/F	13.6	P: 500 mg/dl; RBC: 5–8; WBC: 15–20	1.7	Uraemic syndrome	N.A.	Transplanted, after chronic dialysis
16	44/M	1.3	P: 220 mg/dl; RBC: 3–5; hyaline casts	2.7	Pulmonary haemorrhage, non–nephrotic proteinuria	p-ANCA	After 2 years, stable serum creatinine (1.0 mg/dl)
17	66/M	6.9	P: 500 mg/dl; RBC: >100; granular, waxy, hyaline, WBC, RBC casts; oval fat bodies	1.6	RPGN Uraemic syndrome	(–)	Unknown
18	75/F	3.3	P: 300 mg/dl; RBC: >100	0.5	RPGN Pulmonary haemorrhage	p-ANCA	Unknown
19	55/F	9.9	P: 110 mg/dl; RBC: >100	1.7	RPGN	p-ANCA	Unknown
20	43/M	4.7	P: 500 mg/dl; RBC: >100; granular casts; oval fat bodies	9.0	Nephrotic syndrome	N.A.	Unknown
21	34/F	0.9	P: 310 mg/dl; RBC: >100	6.0	Nephritic syndrome	N.A.	Unknown
22	14/F	3.2	P: 150 mg/dl; RBC: >100	1.7	RPGN	p-ANCA	Unknown
23	62/F	3.2	P: 240 mg/dl; RBC: >100	1.9	RPGN Pulmonary haemorrhage	p-ANCA	Unknown
24	38/F	11.1	P: 60 mg/dl; RBC: >100; WBC: 3–5; granular, broad, waxy, RBC casts	1.0	Uraemic syndrome	p-ANCA	After 2 years, on chronic dialysis

(*continued*)

Table 1. Continued

Patient	Age (year)/ Gender	Initial S_{Cr} (mg/dl)	Urinary findings	Proteinuria (g/24 h)	Clinical features	ANCA	Follow-up
25	27/M	18.6	P: 300 mg/dl; RBC: 21–23; WBC: 2–3; granular, hyaline, RBC casts	3.2	RPGN	(–)	On chronic dialysis
26	65/F	5.6	P: 25 mg/dl; RBC: 2–4	1.0	RPGN	p-ANCA	On chronic dialysis
27	51/F	9.2	P: (+); RBC: (+)	4.3	RPGN Oligoarthritis	p-ANCA	On chronic dialysis
28	69/F	4.9	P: 60 mg/dl; RBC: (+)	1.2	RPGN Pulmonary haemorrhage	p-ANCA	On chronic dialysis
29	18/F	7.3	P: 200 mg/dl; RBC: >100	2.8	RPGN Nephrotic proteinuria	N.A.	Unknown
30	77/M	17.7	P: 500 mg/dl; RBC: >100	3.0	RPGN Pulmonary haemorrhage	p-ANCA	Death on chronic dialysis

S_{Cr}: serum creatinine, M: males, F: females, P: proteinuria, RBC: red blood cells, WBC: white blood cells, N.A.: not available.

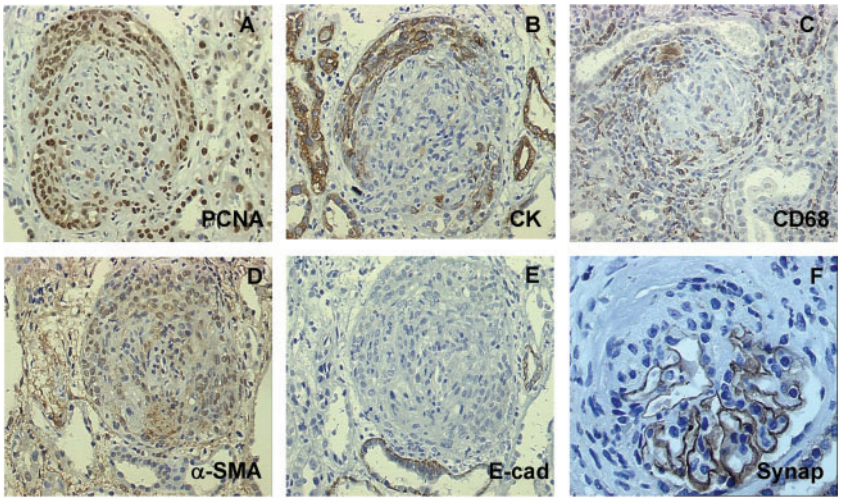

Fig. 1. Immunohistochemistry (IMH) for PCNA, cytokeratin (CK), CD68, α-SMA, E-cadherine and synaptopodin, in glomerular crescents of patients with pauci-immune rapidly progressive GN. Many crescentic parietal epithelial cells (PECs) strongly expressed PCNA (**A**). Cytokeratine (CK) was strongly expressed in the majority of these proliferating crescentic PECs, as well as in the tubular epithelial cells. (**B**). Macrophages (CD68 (+) cells) are identified in these cellular crescents and also as interstitial infiltrating cells (**C**). α-SMA (+) cells were found in crescentic lesions, as well as in periglomerular interstitial fibrosis (**D**). Most of these proliferating crescentic PECs are negative for E-cadherin, that is preserved in some of the epithelial tubular cells (Case 20) (**E**). In this fibrocellular crescent, no cell expressed synaptopodin, a podocyte marker (Case 29) (**F**). (magnification ×200).

TGF-β and gremlin mRNA expression by ISH in serial sections of some biopsies. TGF-β mRNA was not detected by ISH in normal renal tissue (data not shown), however, there was a marked increase in association with glomerular crescent formation. As it is illustrated in Figure 3A and B (case 7), we observed a strong co-localization of TGF-β and gremlin expression in the crescentic cells. Moreover, we also observed a strong co-localization of TGF-β and gremlin on the tubular epithelial cells as it is shown in Figures 3D and E (case 24).

In addition, in cultured human monocyte cells, we have observed that treatment with TGF-β increased gremlin production in a dose-dependent way after 18 h (data not shown) remaining elevated until 24 h (Figure 6). However, in these cells TGF-β did not increase CTGF production (data not shown). This result suggests that the main inducer of gremlin in crescentic, and interstitial cells could be TGF-β, moreover, considering the participation of monocytic cells in the crescents formation and into the interstitial cells.

As TGF-β activate a unique signal transduction pathway acting through the Smad family of proteins, we studied the expression of activated Smad by south-western histochemistry and IMH. In Figure 3C, we observed a definitive nuclear pSmad2 activation by IMH in the crescentic glomerular cells. In addition, we observed the same tubular cells on serial sections, positive for gremlin mRNA (Figure 4A), and activated pSmad2 by IMH (Figure 4B) (case 24).

Since the fibrogenic effects of TGF-β are partially mediated by CTGF and this fibrogenic factor

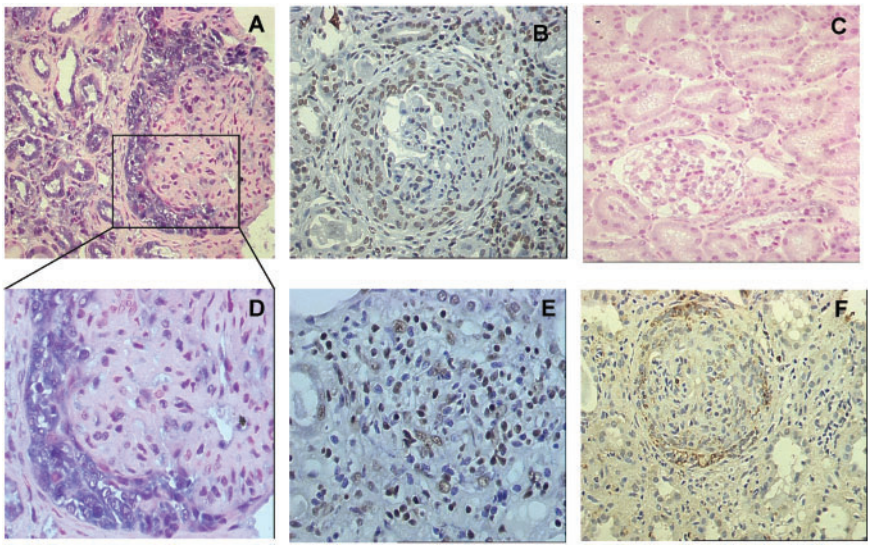

Fig. 2. *In-situ* hybridization (ISH) and IMH demonstrating gremlin mRNA and gremlin protein in crescentic PECs and tubular epithelial cells of patients with pauci-immune crescentic GN. In comparison with normal renal tissue in which there is no expression of gremlin mRNA (**C**), proliferating PECs of glomerular crescents show a strong expression of gremlin mRNA by ISH (**A**–**D**) (case 7) (magnification ×200 and ×400); and gremlin protein expression by IMH (case 7) (**B**) (magnification ×200); immune competent infiltrating interstitial cells are also strongly positive for gremlin staining (**E**); and CTGF was also expressed in these glomerular crescentic cells. (case 20) (**F**).

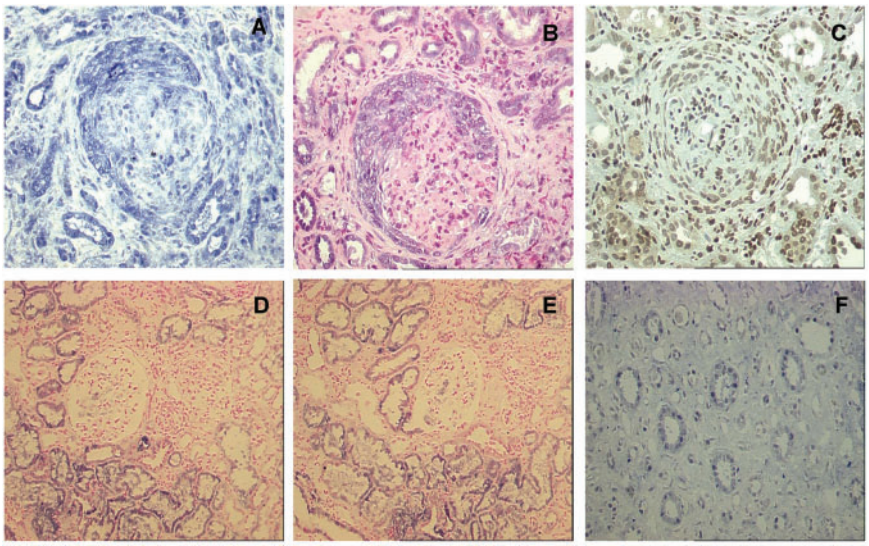

Fig. 3. Co-expression of TGF-β mRNA, gremlin mRNA and Smads in glomerular crescentic cells and tubular cells of patients with RPGN. Strong expression of TGF-β mRNA in proliferating crescentic cells by ISH (**A**) and gremlin mRNA in sections of the same biopsy (**B**). Activated pSmad2 was also observed by IMH in the crescentic glomerular, tubular and infiltrating interstitial cells (**C**) (case 7). On serial sections we observed tubular TGF-β mRNA transcription (**D**) and gremlin mRNA expression (**E**), on the same tubuli (case 16). Smad3 translocated to the nucleus is demonstrated by SWH on tubular cells (**F**) (case 24) (magnification ×200).

participates in the scar formation of crescentic glomerulonephritis, we decided to study the expression of CTGF in these samples. As we denoted in Figure 2F, there was a strong up-regulation of CTGF in crescentic glomerular cells, capsular adhesions and periglomerular fibrosis, as well as in tubular epithelial cells. In addition, since gremlin is a BMP-7 antagonist, we studied their expression by IMH (Figure 4); BMP-7, predominantly expressed in the luminal side of the collecting duct and distal tubular epithelial cells in the normal kidney, was significantly decreased in tubular cells of patients with crescentic GN, and in close relation with the tubular-interstitial involvement (data not shown).

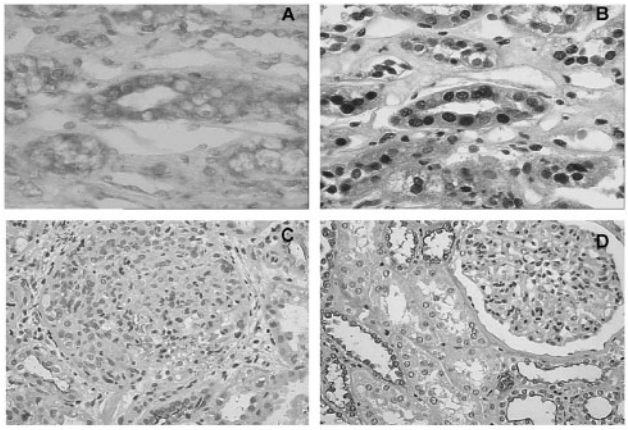

Fig. 4. Co-localization of gremlin and Smad in tubular cells and BMP-7 expression in pauci-immune GN. *In situ* hybridization and immunohistochemistry demonstrating co-localization of tubular gremlin mRNA (**A**) and pSmad2 (**B**) on the same tubular cells. Serial sections of case 24 (magnification 400×). BMP-7 normally expressed in the luminal side of distal tubules (**D**), was significantly decreased in renal sections of patients with pauci-immune RPGN (C).

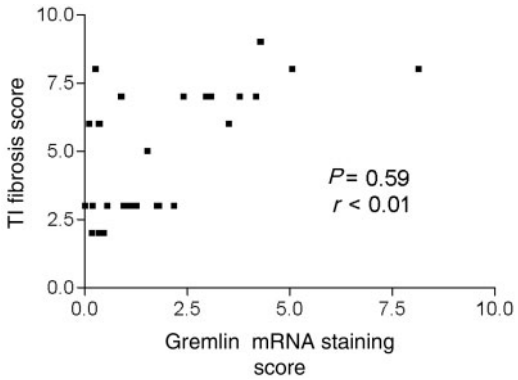

Fig. 5. Correlation between tubule interstitial gremlin mRNA expression and tubule interstitial fibrosis. Gremlin expression correlates with fibrosis score ($r = 0.59$; $P < 0.01$). Points represent individual values for patients with pauci immune crescentic GN.

Discussion

Glomerular cellular crescents consist of parietal epithelial cells (PECs) and macrophages, which can undergo an irreversible process of fibrous organization. Epithelial-mesenchymal transition (EMT) is the term to describe this conversion [5] and plays an important role in the genesis of fibroblasts in interstitial renal fibrosis [13]. A more specific Bowman's epithelial-mesenchymal transition participating in the formation and evolution of glomerular crescents, has been also proposed [7–9].

This study provides the first phenotypic and morphological evidence that gremlin could participate in glomerular crescent formation. In aggregate, these results indicate that the developmental gene gremlin re-emerges in the context of tubulointerstitial fibrosis and

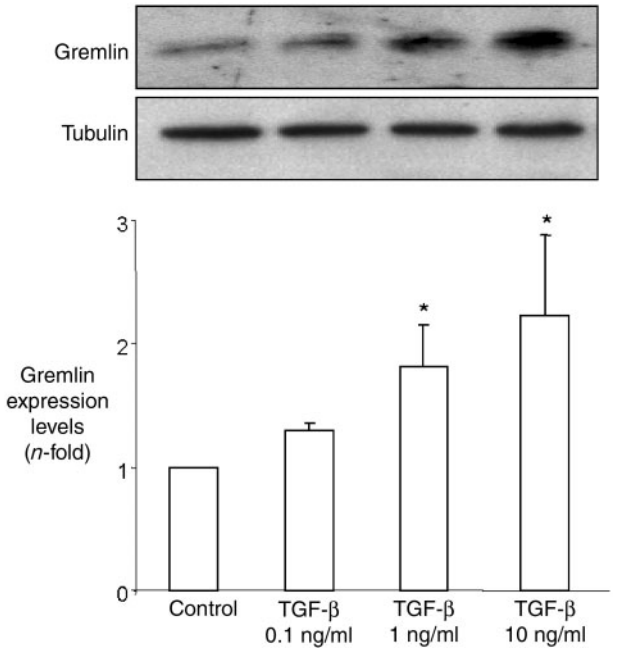

Fig. 6. In human monocyte cells TGF-β increases gremlin expression in a dose dependent way. Cultured human monocyte cells were stimulated with TGF-β (range 0, 1–10 ng/ml) for 24 h. Results of total gremlin production were obtained from densitometric analysis and expressed as ratio Gremlin/Tubuline as *n*-fold over control. Figure shows in the top panel a representative western blot and in the bottom panel, data of gremlin production as mean ± SEM of 4–7 independent experiments. $^{*}P < 0.05$ *vs* control.

suggests a role for TFG-β as an inducer of gremlin expression in this context.

Tubular EMT is an orchestrated, highly regulated process involving four key steps: (i) loss of epithelial cell adhesion by suppression of E-cadherin expression, (ii) *de novo* α-smooth muscle actin expression and actin reorganization, (iii) disruption of the tubular basement membrane and (iv) enhanced cell migration and invasion. TGF-β as a sole factor is capable of inducing epithelial cells to undergo all four steps [13]. In this study, we have observed that PECs lost expression of the epithelial marker, E-cadherin and some cells co-expressed CK and α-SMA suggesting a transitional phase in the dynamic phenomenon of EMT. In addition, cellular crescents contained small numbers of α-SMA-positive myofibroblasts, that become the dominant population in fibrocellular crescents, in which CTGF appears.

The findings showing PCNA-positive crescentic cells, the majority of them dysregulated PECs that become negative for CK, followed by some PECs still positive for CK, macrophagic cells and myofibroblasts, extend and confirm the observations of EMT in pauci-immune crescentic GN reported by Bariety [9].

Recently, we have reported that gremlin mRNA levels by real-time polymerase chain reaction, were significantly elevated in biopsy specimens with diabetic nephropathy compared with normal tissue [4]. Gremlin expression was not increased in specimens of either

minimal change disease or IgA nephropathy. Interestingly, gremlin mRNA was increased in some samples of crescentic nephritis (data not shown), and in diabetic specimens there was a direct correlation between gremlin mRNA levels and tubulointerstitial fibrosis [4], supporting the present data in which we have observed a strong gremlin mRNA and protein expression in the glomerular and interstitial compartment of specimens with crescentic GN. Co-localization of gremlin and TGF-β1 expression in crescentic cells suggests that TGF-β1, may also modulate gremlin expression in the glomeruli; this hypothesis is strengthened further because co-localization of expression is also evident in tubular cells, particularly in areas where tubulointerstitial fibrosis has developed. In addition, we have shown that gremlin expression is induced *in vitro* by TGF-β in human monocytic cells, extending those observations performed on renal proximal tubule cells undergoing transdifferentiation to a fibroblast phenotype [3], and supporting a role for gremlin in the pathogenesis of tubulointerstitial fibrosis.

As noted, gremlin is a BMP antagonist, by heterodimerization with BMP 2, 4 and 7 and thereby prevents receptor binding [2]. Recently, it has been reported that BMP-7 reverses TGF-β1-induced epithelial to mesenchymal transition by reinduction of E-cadherin, a key epithelial cell adhesion molecule [14]. In this context it is very intriguing that administration of the gremlin ligand BMP-7 is protective in models of progressive renal disease [15], raising the possibility that gremlin may even be a therapeutic target. Actually, we have observed that the tubular BMP-7 expression is significantly decreased in biopsies of patients with progressive crescentic GN in comparison with normal renal tissue, and these findings are coincident with the loss of tubular BMP-7 and the increase in gremlin observed in experimental diabetic nephropathy [15].

TGF-β is known to mediate its fibrotic effects by activating the receptor-associated Smads and the discovery of the TGF-β/Smad signalling pathway has allowed to study the intracellular mechanisms of TGF-β in EMT [16]. The Smads are divided into three categories. The first category consists of the receptor-activated or pathway-restricted Smads (R-Smads), that include Smad2 and Smad3, and are activated by relatively specific TGF-β receptor ligands. The phosphorylated R-Smads associate to form a heteromultimer that includes the second type of Smad, the common-partner Smad 4. The complex is then translocated to the nucleus, where it can regulate target gene transcription. A third category comprises of the inhibitory Smads (Smad 6, Smad 7) [17] that appear to function as competitive inhibitors of Smad activation.

The complementary technique of SWH performed in this study using a labelled Smad-binding element demonstrated increased binding of nuclear proteins to the Smad-binding element, indicating active signalling downstream of the TGF-β stimulus. The observed co-localization of TGF-β, and activated Smad2 and Smad3 translocated into the nucleus, confirm the TGF-β/Smad-signalling pathway in the EMT. Furthermore, the co-localization of the BMP antagonist, gremlin, on the same glomerular and tubular cells suggests that the gremlin induction by TGF-β could be occurring through the TGF-β/Smad-signalling pathway.

Recent results suggest that CTGF may play a crucial role in the renal EMT and the subsequent deposition/degradation process of ECM during tubulointerstitial fibrosis [18]. In this context, the present study confirms observations of Ito *et al*. [19] that first demonstrated that CTGF is expressed in the crescents of human GN, and observations of Kanemoto *et al*. [20], that showed the participation of CTGF in scar formation of crescentic GN. Interestingly, CTGF mRNA was localized in the PECs, but not in macrophages, suggesting that inflammatory cells are not a major source of CTGF in cellular crescents [20]. Our *in vitro* results confirm that monocytes are not a source of CTGF when stimulated by TGF-β. Instead, gremlin is induced.

The regulatory mechanisms for CTGF and gremlin in cellular crescents need to be determined, although it is well known that CTGF play an important role in mediating the profibrotic effects of TGF-β [18]. Of interest, on the other hand, in vitro studies performed in cultured human proximal tubular epithelial cells have shown that TGF-β1 upregulated CTGF gene expression, preceding that of α-SMA and fibronectin. The α-SMA was significantly inhibited by the CTGF antisense oligodeoxynucleotide [18].

In conclusion, we have reported the presence of gremlin in crescentic proliferating cells, the majority PECs, which are evolving to a myofibroblast phenotype, that suggests gremlin as a downstream mediator of TGF-β either acting as an inhibitory trap protein for BMP-7 creating a profibrotic positive loop or directly by promoting the transdifferentiation of epithelial cells. Studies are in progress to demonstrate the exact role of gremlin in the EMT.

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Conflict of interest statement. None declared.

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COMPLICATIONS
Nephropathic

Gremlin: A Novel Mediator of Epithelial Mesenchymal Transition and Fibrosis in Chronic Allograft Nephropathy

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ABSTRACT

Background. Chronic allograft nephropathy (CAN) is the most frequent cause of chronic dysfunction and late loss of renal allografts. Epithelial mesenchymal transition (EMT) has been identified as responsible for the presence of activated interstitial fibroblasts (myofibroblasts) and transforming growth factor beta (TGF-β)/Smad is the key signaling mediator. It has been proposed that the bone morphogenetic protein 7 (BMP-7) antagonist, Gremlin, could participate in EMT, as a downstream mediator of TGF-β.

Methods. We evaluated 33 renal allograft biopsies, 16 of which showed CAN, versus 17 controls. By in situ hybridization we studied the expression of TGF-β and Gremlin mRNA. Gremlin, BMP-7, E-cadherin, and α-smooth muscle actin (α-SMA) proteins were evaluated by immunohistochemistry and Smad3 activation by Southwestern. In cultured human tubuloepithelial cells (HK2 cell line), Gremlin induction by TGF-β was studied by confocal microscopy.

Results. Among renal biopsies of transplanted patients with CAN, we detected up-regulation of TGF-β in colocalization with Gremlin (RNA and protein), mainly in areas of tubulointerstitial fibrosis. In the same tubules, we observed decreased expression of E-cadherin and induction of vimentin and α-SMA. BMP-7 was significantly decreased in the CAN biopsies. In addition, HK2 stimulated with TGF-β (1 ng/mL) induced Gremlin production at 72 hours.

Conclusion. We postulated that Gremlin is a downstream mediator of TGF-β, suggesting a role for Gremlin in EMT observed in CAN.

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CHRONIC ALLOGRAFT NEPHROPATHY (CAN) is the main cause of renal allograft failure following the first year after transplantation. This multifactorial process is defined as a renal allograft dysfunction, occurring at least 3 months posttransplant, in the absence of active acute rejection, drug toxicity (principally calcineurin inhibitors), or other diseases. The pathological changes of chronic renal allograft nephropathy involve all parts of the renal parenchyma, including the blood vessels, glomeruli, interstitium, and tubules. Tubulointerstitial fibrosis is the main factor involved in the loss of renal function in CAN.

Epithelial-mesenchymal transition (EMT) is an important process during renal fibrosis by which renal tubular cells lose their epithelial phenotype and acquire new characteristic features of mesenchyme.[1–3] Transforming growth factor-β (TGF-β) is a key fibrogenic factor that regulates epithelial to myofibroblast transition in renal tubular cells.[4,5]

Gremlin, an antagonist of bone morphogenetic protein 7 (BMP-7) with a crucial role in normal development,[6–8] is overexpressed in adult pathological processes, such as diabetic nephropathy (DN)[9,10] and pauci-immune glomerulonephritis, as recently reported by our group.[11] The upregulation of Gremlin in DN has been correlated with TGF-β expression, both in animals and humans. It has been suggested that Gremlin could participate in EMT, acting as a downstream mediator of TGF-β.[12] The aim of our work was to study the expression of Gremlin and TGF-β in patients with CAN, and their relationship to the development of EMT.

MATERIALS AND METHODS

Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation. After obtaining patient consent and approval by the local hospital ethics committee, renal biopsies from 33 transplanted patients were studied; 16 with CAN and 17 controls—five acute tubular necrosis, four acute rejection, eight unspecific lesions.

Assessment of the Pattern of Gremlin Expression by in situ Hybridization

In situ hybridization (ISH) was performed as described previously for antisense TGF-β probe (R&D Systems, Minneapolis, Minn, USA),[13] and with the following modifications for biotin-labeled human Gremlin probes (Invitrogen, Carlsbad, Calif, USA). The Gremlin probes were: 478 antisense 5′-TGAAAGGAACCTTC-CTCCTTCC3′ 2416 antisense 5′-ATGGGAGAGCACTGGAT-CAAAA-3′ and 3553 antisense 5′-CAGGCACTGACTCAGGAA-GACA-3′. For Gremlin analysis, pretreatment with an endogenous biotin blocking system (Dako Co, Carpinteria, Calif, USA) was performed prior to proteinase K digestion. The sections were incubated with a prehybridization solution (Dako, mRNA ISH solution) for 60 minutes at 37°C and with the antisense probe overnight at 37°C. The slides for Gremlin were washed with 2 × SSC and 1 × SSC for 10 minutes at room temperature and then with 0.5 × SCC for 20 minutes at 37°C.

Detection was performed with avidin-alkaline phosphatase conjugate (Dako) for 30 minutes at room temperature, washed 5 minutes with 1 × TBS and using NBT-BCIP as the enzyme substrate for 120 minutes at 37°C (R&D Systems). Tissues were then dehydrated in an ethanol series and mounted in Canadian balsam (Polysciences Inc, Warrington, Pa, USA).

The specificity of the reaction was confirmed: (1) by demonstrating the disappearance of hybridization signal when RNAse (100 ug/mL; Sigma Chemicals Co, St Louis, Mo, USA) was added in 0.05 mol/L Tris after the digestion with proteinase K; (2) by the use of a sense probe (R&D Systems); (3) with a negative control (Plasmid DNA) (Dako); and (4) without probe. For Gremlin ISH slides, Dako nuclear fast red was used for 10 minutes.

Immunohistochemistry

For light microscopy, kidney tissues were fixed in 4% buffered formalin, or Bouin, dehydrated and embedded in paraffin by conventional techniques. Sections were stained with hematoxylin and eosin, periodic acid-Schiff, and methenamine silver. Paraffin-embedded biopsy specimens were used to detect Gremlin, α-smooth muscle actin (α-SMA), vimentin, E-cadherin.

The following primary antibodies were employed: rabbit polyclonal anti-Gremlin (ABGENT, AP6133a, San Diego, Calif, USA); mouse anti-human α-SMA clone 1A4 (Dako); mouse anti-human E-cadherin clone 36B5 (Novocastra, Newcastle, UK); and anti-human vimentin (BD Pharmygen).

Briefly, 5-μm-thick Bouin- or formalin-fixed renal sections were deparaffinized through xylene, alcohol, and distilled water. Endogenous peroxidase was blocked by 3% H_2O_2 for 15 minutes before the sections were treated in a microwave oven in a solution of 0.1 mmol/L citrate buffer (pH 6.0 10 minutes) or an 1 mmol/L ethylenediamine tetraacetic acid (EDTA) buffer (pH 8.0) for synaptopodin detection. After blocking, the sections were incubated overnight at 4°C with the specific primary antibody. The sections were then incubated with the corresponding biotinylated secondary antibodies for 30 minutes at 22°C. After three rinses in Tris saline buffer, they were incubated with streptavidin-peroxidase (Dako) 1/1000 for 30 minutes. Color was developed with substrate (Dako) and then counterstained with hematoxylin, dehydrated, and mounted with Canadian balsam (Polysciences, Inc). The specificity was checked by omission of primary antibodies and use of nonimmune sera.

Southwestern Histochemistry

Briefly, complementary oligonucleotides containing a Smad binding consensus sequence were synthesized by Invitrogen as follows: 5′-GAGTATGTCTAGACTGACAATGTAC-3′. After annealing with their complementary DNA (80°C during 2 minutes), the probe was labeled with digoxigenin (DIG oligonucleotide 3-end labeling kit; Boehringer Mannheim, Mannheim, Germany). Paraffin-embedded kidney sections were dewaxed, rehydrated, and incubated with 5 mmol/L levamisole (Sigma Chemical Co) to inhibit endogenous alkaline phosphatase, and fixed with 0.2% p-formaldehyde for 30 minutes at 28°C. Sections were subsequently digested with pepsin A (433 U/mg; Sigma), washed twice with buffer 1 (10 mmol/L HEPES, 40 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L DTT, 1 mmol/L EDTA, 0.25% bovine serum albumin (BSA), pH 7.4), and then with 0.1 mg/mL DNAse I, washed once with buffer 2 (10 mmol/L HEPES, 40 mmol/L NaCl, 1 mmol/L DTT, 10 mmol/L EDTA, 0.25% BSA, pH 7.4) to stop the reaction. The labeled probe (100 pmol/L) diluted in buffer 1 containing 0.5 mg/mL poly (dl-dC; Pharmacia LKB, Piscataway, NJ, USA) was applied overnight at 37°C. After washing, sections were incubated for 1 hour in

blocking solution (0.01× SSC, 0.01% SDS, 0.03% Tween 20, 0.1 mol/L maleic acid, 0.15 mol/L NaCl, pH 7.5), and with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:250 in blocking solution; Boehringer Mannheim) overnight at 4°C. The color reaction was developed using NBT/BCIP (Dako).

Immunohistochemistry Quantification

The labeled surface area was evaluated by quantitative image analysis using a KS 300 imaging system 3.0 (Zeiss, München-Hallbergmoos, Germany). For each sample, the mean staining area was obtained by analysis of 20 fields (20×). Quantification was done twice, independently, and interassay variations were not significant. The staining score is expressed as percentage/mm^2.

Tubulointerstitial cell infiltration and interstitial fibrosis were classified into four groups according to their extent and the presence of tubular atrophy and degeneration: (1) normal, (2) involvement up to 25% of the cortex, (3) involvement of 26% to 50% of cortex, and (4) extensive damage involving more than 50% of the cortex.

Cell Cultures

Human renal proximal tubuloepithelial cells (HK-2) were grown in RPMI with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin, ITS and hydrocortisone in 5% CO_2 at 37°C. At 60% to 70% confluence, cells were growth-arrested in serum-free medium for 24 hours before the experiments. The experiments were performed in serum-free medium. TGF-β was added at dose of 1 ng/mL. Cells growing in cover slips were fixed in Merck fix (Merck), treated with 0.1% Triton-X100, incubated with Gremlin antibody (ABGENT AP6133A, San Diego, Calif, USA) followed by FITC-conjugated antibody. Samples were mounted in Mowiol 40–88 (Sigma) and examined by a laser scanning confocal microscope (Leika). The experiments were done with three different cell culture preparations.

Statistical Analysis

The statistical analysis was performed with the GraphPad Instat (GraphPad Software, San Diego, Calif, USA). The results of the clinical data are expressed as mean values ± SD. A Spearman correlation was used to correlate tubulointerstitial Gremlin expression and tubulointerstitial cell infiltration and fibrosis. Pearson correlation was used to correlate Gremlin mRNA and protein expression.

RESULTS

Clinical Findings of Patients Studied

Table 1 illustrates the clinical and biological data of the patients with CAN at the time of the biopsy. All of them had received steroids, cyclosporine, and azathioprine. Four out of 16 were transplanted with living related donors. The biopsy was prescribed by renal functional deterioration with nephrotic range proteinuria among 3 out of 16 cases.

Evidence of EMT Changes in Patients With CAN

By immunohistochemistry, we evaluated the expression of E-cadherin, a tubular epithelial marker found within adherent-type junctions. E-cadherin plays an essential role to preserve the structural integrity of renal epithelia; it is lost during tubular EMT. We also evaluated the expression of mesenchymal markers such as α-SMA and vimentin. These proteins are important to define the morphology of the transformed cells and their capacity for contractility, migration, and invasion.

Among control biopsies, we observed a marked expression of E-cadherin in the tubular epithelium with no staining for mesenchymal markers in tubular cells. These findings were correlated with the integrity of the tubular epithelium.

In biopsies of patients undergoing CAN, we observed a loss of the expression of E-cadherin in the tubular epithelium and a strong up-regulation of mesenchymal markers α-SMA and vimentin in the tubular and interstitial infiltrating cells.

These findings supported the presence of EMT in the tubular epithelial cells of patients undergoing CAN (Fig 1).

Table 1. Clincal and Biological Data From the 16 Patients With Chronic Allograft Nephropathy

Patients	Age (y)	Gender	Scr (mg/dL)	Proteinuria (g/24 h)	Immunosuppression	Donor
1	44	M	2.15	4.5	Aza, CsA, Prd	R
2	31	F	4.65	0	Aza, CsA, Prd	R
3	58	M	2.3	0	Aza, CsA, Prd	R
4	50	F	3.9	0.9	Aza, CsA, Prd	C
5	52	M	3.5	NA	Aza, CsA, Prd	C
6	48	M	1.4	1.3	Aza, CsA, Prd	R
7	30	F	2.0	0.9	Aza, CsA, Prd	C
8	46	M	5.3	2.2	Aza, CsA, Prd	C
9	53	F	7.6	2.2	Aza, CsA, Prd	C
10	58	M	1.8	6.7	Aza, CsA, Prd	C
11	27	M	3.5	0	Aza, CsA, Prd	C
12	55	M	5.2	0	Aza, CsA, Prd	C
13	46	M	3.5	3.0	Aza, CsA, Prd	C
14	51	M	0.8	2.6	Aza, CsA, Prd	C
15	59	M	3.1	0	Aza, CsA, Prd	C
16	40	M	1.9	0	Aza, CsA, Prd	C

SCr, serum creatinine; NA, not aviable; AZA, azathioprine; CsA, cyclosporine; Prd, prednisone; R, related; C, cadaver.

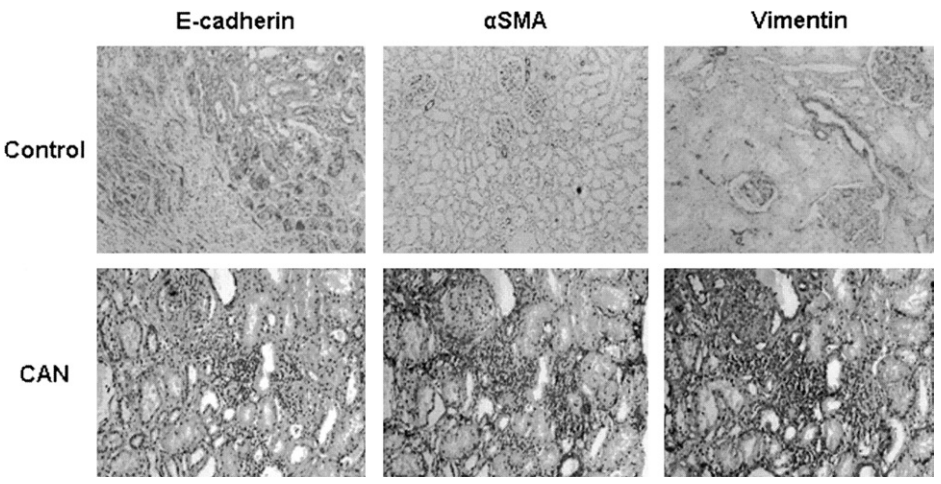

Fig 1. In comparison with normal renal tissue in which there is a strong staining for E-cadherin in almost all the tubular cells and constitutive expression of α-SMA and vimentin, in renal sections of patients with CAN there was a significant decreased expression of tubular E-cadherin and a strong induction in α-SMA and vimentin, mainly in areas of tubule interstitial fibrosis. Immunohistochemestry, magnification $\times$200. (Case 7.)

Coexpression of TGF-β and Gremlin mRNA

By in situ hybridization of serial sections of biopsies of patients with CAN, we evaluated the mRNA expression of TGF-β, the main promoter of EMT in tubular cells, and Gremlin. As the main signal transduction pathway of TGF-β is the Smad family of proteins, we also studied the expression of activated Smad by Southwestern histochemistry.

We observed strong coexpression of TGF-β and Gremlin mRNA in tubular cells as well as Smad translocation to the nucleus (Fig 2). In renal biopsies of control patients, we did not detect the expression of Gremlin (data not shown). Control biopsies did not show elements of fibrosis.

Gremlin Expression Is Correlated With Tubulointerstitial Fibrosis

By Spearman test, we evaluated the correlation between Gremlin mRNA expression and tubulointerstitial fibrosis. Gremlin protein and mRNA were mainly detected in areas with interstitial cell infiltration and interstitial fibrosis. We observed a strong correlation ($r = .61$, $P < .01$) between Gremlin expression and tubulointerstitial fibrosis (Fig 3).

TGF-β Induces Expression of Gremlin in Human Tubuloepithelial Cells

In human tubuloepithelial cells, incubation with TGF-β for 3 days caused phenotypic conversion. The transformed cells lost their typical cobblestone pattern of an epithelial monolayer, displaying spindle-shape, fibroblast-like morphology as assessed by phase contrast microscopy (not shown). By immunohistochemistry, we evaluated the expression of Gremlin protein in cells stimulated with TGF-β. There was a strong expression of Gremlin in stimulated cells compared with almost no staining in unstimulated tubuloepithelial cells (Fig 4).

DISCUSSION

Renal allograft failure is a common cause of end-stage renal disease. The main cause of renal failure after the first year of transplant is a multifactorial and not well-understood entity called *chronic allograft nephropathy*. Tubulointerstitial fibrosis is the main factor involved in the loss of renal function in CAN. Growing evidence suggests that EMT plays a key role in renal tubulointerstitial fibrosis.[1–3,14] It has been reported that the process of EMT requires four steps: (1) loss of epithelial adhesion properties, (2) de novo

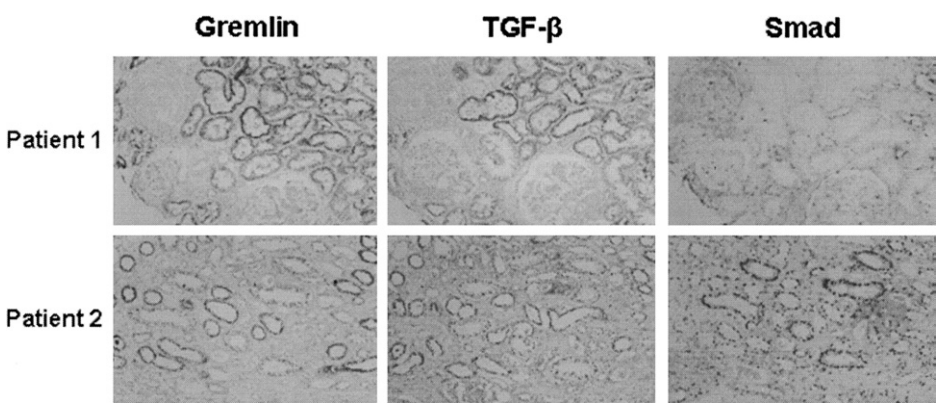

Fig 2. Coexpression of TGF-β mRNA, Gremlin mRNA, and Smads in serial sections of biopsies in patients with chronic allograft nephropathy. On serial sections we observed tubular TGF-β mRNA transcription and Gremlin mRNA expression on the same tubuli. In situ hybridization, magnification $\times$200. (Case 16.) Smad3 translocated to the nucleus is demonstrated by Southwestern hystochemistry on the same tubular cells. Magnification $\times$200. (Case 16.)

expression of mesenchymal markers and actin reorganization, (3) disruption of tubular basal membrane, and (4) enhanced cell migration and invasion.[15] In this study, we observed that biopsies of patients undergoing CAN show diminished expression of E-cadherin and of de novo expression of mesenchymal markers vimentin and α-SMA, suggesting that the tubular cells lose their epithelial phenotype, acquiring the new elements characteristics of mesenchymal elements.

TGF-β, a key factor in EMT, is well known to be a profibrogenic cytokine capable of inducing tubular epithelial cells to undergo all four steps of EMT,[5] but because of its pleiotropic effects, it is not an ideal therapeutic target.

Gremlin is a 184-amino-acid protein and a member of cysteine knot super family, which serves as an antagonist of bone morphogenetic proteins (BMPs) with an important role in normal development.[16] Gremlin influences diverse processes in growth, differentiation, and development by heterodimerization with BMPs, thereby inhibiting the ability of these ligands to bind to their receptors.[6–8]

Epithelial-mesenchymal feedback signaling is the key to diverse organogenesis processes such as limb bud development and branching morphogenesis in kidney and lung rudiments. Gremlin is essential to initiate these epithelial-mesenchymal signaling interactions during limb and metanephric kidney organogenesis.

Gremlin was first identified and named as Induced in High Glucose 2 in mesangial cells exposed to high extracellular glucose in vitro.[10] Increased Gremlin expression has also been observed in streptozotocin-induced diabetic nephropathy and the 5/6 nephrectomy model of glomerular hypertension in vivo. TGF-β added to human mesangial cells increased Gremlin expression, while the stimulatory effect of high glucose on Gremlin expression was attenuated by the addition of anti-TGF-β antibody.[12] This evidence suggested that Gremlin is induced by TGF-β in diabetic nephropathy. We have also observed Gremlin expression in kidney biopsies from patients with diabetic nephropathy. This expression was most pronounced in areas of interstitial fibrosis and was colocalized with TGF-β.[17] In contrast, normal kidney and biopsies from patients with nonscarring renal disorders, such as minimal change disease, displayed significantly less or no expression of this protein.

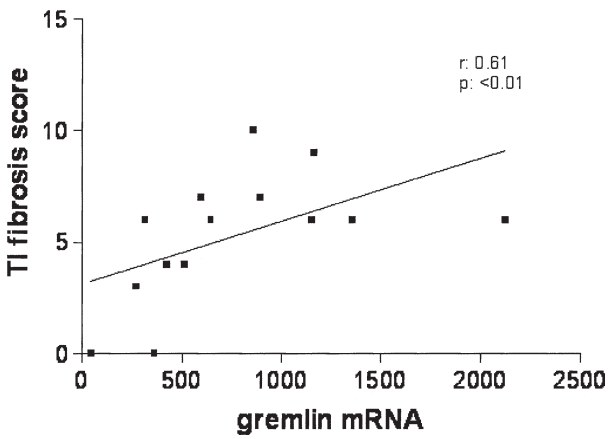

Fig 3. Spearman correlation between Gremlin mRNA expression and tubulointerstitial fibrosis in chronic allograft nephropathy. Gremlin expression correlates with fibrosis score ($r = .61$, $P < .01$). Points represent individual values for patients with chronic allograft nephropathy.

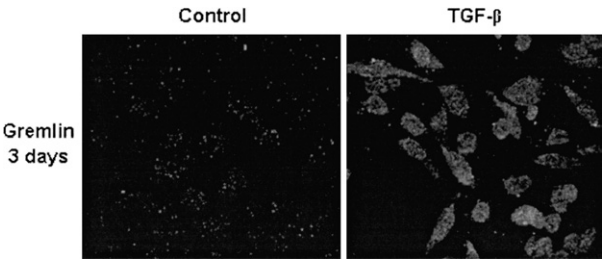

Fig 4. In cultured human tubuloepithelial cells (HK2 cell line), Gremlin induction by TGF-β was studied by confocal microscopy. Stimulation of tubular epithelial cells with TGF-β (1 ng/mL) induces strong overexpression of Gremlin at 72 hours.

EMT also occurs in parietal epithelial glomerular cells,[18] leading to crescent formation. Recently we have report the presence of Gremlin in crescentic proliferating cells and tubular cells of renal biopsies from patients undergoing pauci-immune glomerulonephritis.[11] This Gremlin expression was colocalized with TGF-β, suggesting gremlin to be a downstream mediator of TGF-β, either acting as an inhibitory trap protein for BMP-7 creating a profibrotic positive loop or directly by promoting the transdifferentiation of epithelial cells. All these initial studies suggested that Gremlin overexpression contributes to transdifferentiation of renal epithelial cells to a more fibroblast-like phenotype.

In this study we evaluated the expression of Gremlin in kidney biopsies from patients undergoing CAN. We observed strong expression of Gremlin in tubular cells; it colocalized with TGF-β. This Gremlin mRNA expression correlated with the degree of tubulointerstitial fibrosis. Renal biopsies of control patients did not present elements of fibrosis and the expression of Gremlin was not detected.

Furthermore, in vitro studies performed in human tubuloepithelial cells undergoing EMT mediated by TGF-β allowed us to observe strong Gremlin expression, thus confirming colocalization between TGF-β and Gremlin observed in renal biopsies in the same tubular cells.

This study provided evidence that Gremlin, a BMP-7 antagonist, may have a role in CAN as a mediator of EMT and fibrosis. These findings support the pathogenic role of Gremlin in human progressive nephropathy and other pathologies that undergo fibrosis, such as idiopathic pulmonary fibrosis[19] and liver fibrosis,[20] and identifies Gremlin as a potential therapeutic target. Further studies are needed to reveal the mechanisms by which Gremlin induces EMT.

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GREMLIN, A BMP-7 ANTAGONIST, IS INDUCED IN HUMAN PROGRESSIVE GLOMERULOPATHIES AND IS ASSOCIATED WITH TUBULOINTERSTITIAL FIBROSIS

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Running title: **Gremlin and renal fibrosis**

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ABSTRACT

Fibrosis is a common feature of chronic kidney diseases that is mediated by matrix-producing myofibroblasts. Epithelial mesenquimal transition (EMT) is increasingly recognized as an integral part of tissue fibrogenesis after injury. The TGF-β/Smad signaling pathway has a key role in EMT. It has been proposed that Gremlin, BMP-7 antagonist, could participate in the process of EMT in diabetic nephropathy and in pauci-immune glomerulonephritis, as a downstream mediator of TGF-β.

The aim of this study was to examine the presence of Gremlin and its correlation with fibrosis and EMT in 125 renal biopsies of patients with a broad group of progressive and non progressive glomerulopathies, evaluating the involvement of the TGF-β/Smad pathway in the process.

We observed a marked induction of Gremlin gene and protein expression in all the progressive glomerulopathies studied. By contrast, in the non progressive nephropathy, minimal change disease, there was no Gremlin detection, as described previously for normal renal tissue. There was a strong correlation between Gremlin mRNA expression and the degree of tubulo-interstitial fibrosis. Gremlin induction was associated with EMT changes, such as, loss of the epithelial marker E-cadherin and gain of the mesenchymal marker α smooth muscle actin. Gremlin expression was found in the glomeruli and in the tubulointerstitial area. We found strong co-localization of Gremlin and TGF-β mRNA in the tubular epithelial cells. Gremlin induction was also associated with Smad activation.

In conclusion, we propose that Gremlin could be a common pathogenic mediator in progressive glomerulopathies including DN, modulating the EMT process through regulation of the TGF-β/Smad signalling pathway.

INTRODUCTION

Many embryological expressed genes regulate morphogenesis and then become quiescent in the normal adult kidney. Recent studies have shown that some developmental genes are reactivated in the adult diseased kidneys.[1] The re-emergence of these genes appears to be linked to tissue repair, but when an imprecise interaction of developmental and inflammatory signals occurs, complete healing is not achieved. Instead, there is a fibrotic process leading to a scar formation. Gremlin was identified as one of the developmental genes induced in cultured human mesangial cells exposed to high glucose and initially known as induced in high glucose-2 (IHG-2).[10] Gremlin is a member of cysteine knot superfamily[2], belongs to a family of bone morphogenetic proteins (BMPs) antagonists and is highly conserved during evolution.[3] Gremlin heterodimerizes with BMPs -2, -4 and -7, preventing their interactions with specific receptors and this capacity is thought to be responsible for the critical role of Gremlin during the process of nephrogenesis, fibrosis, and cancer.[3-7] However, BMP-independent mechanisms may mediate several gremlin intracellular actions, such its ability to suppress tumorigenesis and stimulate endothelial-cell migration.[8,9] Taken together these observations indicate that gremlin may exert multiple functions in different physiopathologic conditions via BMP-dependent and BMP-independent mechanisms.

Several studies have identified the developmentally regulated gene Gremlin as a novel target implicated in diabetic nephropathy (DN).[11-13] Induction of Gremlin was reported in cultured human mesangial cells exposed to high glucose and transforming growth factor β1 (TGF-β1) *in vitro* and kidneys from diabetic rats *in vivo*.[10] A knockout mice heterozygous for grem1 gene deletion (grem1(+/-)) exhibited protection from the progression of diabetic kidney disease in a streptozotocin-induced model of type 1.[14] All these data identify Gremlin as a potential modifier of renal injury in the context of diabetic kidney disease.

Irrespective of the underlying cause, chronic kidney disease is linked with the development of tubulointerstitial fibrosis (TIF), characterized by accumulation of extracellular matrix (ECM). The key cellular mediator of fibrosis is the myofibroblast. In addition to activated local tissue fibroblasts, myofibroblasts can originate from circulating mesenchymal progenitors or from epithelial cells in a process known as epithelial-mesenchymal transition (EMT).[15-17] There is a change in morphology from the cobblestone-like cell morphology typical of an epithelial phenotype to the elongated, fusiform cell sheet characteristic of fibroblasts. The epithelial cells lose polarity and intercellular adhesion and gain mesenchymal properties including motility. During this process epithelial

markers (E-cadherin, cytokeratin) are lost and mesenchymal markers, such as vimentin and α-smooth muscle actin (α-SMA), increase. [15-17]

TGF-β is a profibrotic factor that plays a key role in renal fibrosis. This factor regulates the EMT process, mainly through the activation of the Smad signalling pathway.[18-21] Recent *in vitro* studies developed by our group have shown Gremlin induction by TGF-β in cultured proximal tubulo-epithelial cells and monocytes.[23,24] We also reported the presence of Gremlin in cellular crescents of pauci-inmune glomerulonephritis and in tubulointerstitium of chronic allograft nephropathy, correlated with the degree of TIF and associated with evidence of EMT-related changes.[23,24]

The aim of this work was to examine the presence of Gremlin and its correlation with fibrosis and EMT in a broad group of human nephropathies, evaluating the involvement of the TGF-β/Smad pathway in this process.

MATERIAL AND METHODS

Human renal biopsies

Kidney samples were obtained by percutaneous renal biopsy from patients undergoing diagnostic evaluation at the Division of Nephrology, Austral University, Valdivia, Chile. The samples were studied after obtaining patient consent and the project was approved by the local hospital ethics committee. Renal biopsies from 125 patients were studied, 12 of them with minimal change disease (MCD), 17 focal segmental glomerulosclerosis (FSGS), 33 membranous nephropathy (MN), 10 IgA nephropathy (IgAN), 32 rapidly progressive glomerulonephritis (RPGN), 8 lupus nephritis (LN) and 13 diabetic nephropathy (DN). Paraffin-embedded renal sections were used for detection of gremlin, α-SMA and E-cadherin. The following primary antibodies were employed: rabbit polyclonal anti-gremlin (ABGENT, AP6133a, San Diego CA, USA,); mouse anti-human α-SMA clone 1A4 (Dako) and mouse anti-human E-cadherin clone 36B5 (Novocastra, Newcastle, UK).

Assessment of the pattern of gremlin expression by *In situ* hybridization (ISH).

ISH was performed as described previously for antisense TGF-β probe (R&D Systems, Minneapolis, MN, USA),[28] and with the following modifications for biotin-labeled human gremlin probes (Invitrogen, Carlsbad, CA, USA). The gremlin probes were: 478 antisense 5´-TGAAAGGAACCTTCCTCCTTCC3´ 2416 antisense 5´-ATGGGAGAGCACTGGATCAAAA-3´ and

3553 antisense 5`-CAGGCACTGACTCAGGAAGACA- 3´ For gremlin analysis, pretreatment with endogenous biotin blocking system (Dako Co, Carpinteria, CA, USA) was performed prior to proteinase K digestion. The sections were incubated with a pre-hybridization solution (Dako, mRNA ISH Solution) for 60 min at 37ºC and with the antisense probe overnigth at 37ºC. The slides for gremlin were washed with 2XSSC and 1XSSC for 10 min at room temperature and then with 0.5XSCC for 20 min at 37ºC. Detection was performed with avidin-alkaline phosphatase conjugate (Dako) for 30 min at room temperature, rinsed 5 min with 1XTBS and using NBT-BCIP as the enzyme substrate for 60 min at 37ºC (Dako). Tissues were then dehydrated in ethanol series and mounted in Canadian balsam (Polysciences Inc., Warrington, PA, USA).

The specificity of the reaction was confirmed by: a) demonstrating the disappearance of hybridization signal when RNAse (100 µg/ml) (Sigma Chemicals Co., St Louis, MO, USA) was added in 0.05M Tris after the digestion with proteinase K; b) by the use of a sense probe (R&D Systems); c) with a negative control (Plasmid DNA) (Dako) and d) without probe. For gremlin ISH slides, Dako nuclear fast red was used for 10 minutes.

Statistical analysis

In situ hybridization was quantified by image analysis using a KZ 300 imaging system 3.0 (Zeiss, Munchen-Hallbergmoos, Germany). The staining score is expressed as % stained area, and it was calculated as the ratio of stained area vs. the total field area. For each sample, the mean staining area was obtained by analysis of 20 different fields (x200). In all cases, evaluations were performed by two independent observers in a blinded fashion and the mean score value calculated for each sample.

Tubulointerstitial cell infiltration and interstitial fibrosis (TIF) was classified into four groups according to their extent and the presence of tubular atrophy, and scored between 0-100 % following this criteria: a) normal, b) involvement up to 25% of the cortex, c) involvement of 26 to 50% of cortex, and d) extensive damage involving more than 50% of the cortex.

Differences between groups were assessed by one-way analysis of variance, followed by post-hoc Bonferroni or Dunnett test, or Mann-Whitney test, as appropriate. $P<0.05$ was considered significant. Spearmen test was done to correlate TIF and gremlin mRNA expression. Statistical analysis was conducted using the SPSS statistical software (version 11.0, Chicago, IL).

RESULTS

Renal induction of Gremlin expression was associated with fibrosis in human progressive glomerular diseases

In normal adult kidney, Gremlin is not expressed (neither at gene nor at protein levels).[12] Previous studies have shown induction of Gremlin mRNA in diabetic nephropathy (DN).[11-14] Now, we have evaluated Gremlin gene expression in 125 renal biopsies from patients with different glomerular diseases. These studies included minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), membranous nephropathy (MN), IgA nephropathy (IgAN), rapidly progressive glomerulonephritis (RPGN), lupus nephritis (LN) and DN. In all nephropathies studied, except in MCD, a marked induction of Gremlin gene expression was found (Figure 1). There was also a strong correlation between Gremlin mRNA expression and TIF ($r=0.7$, $p < 0.01$, evaluated by Spearman test).

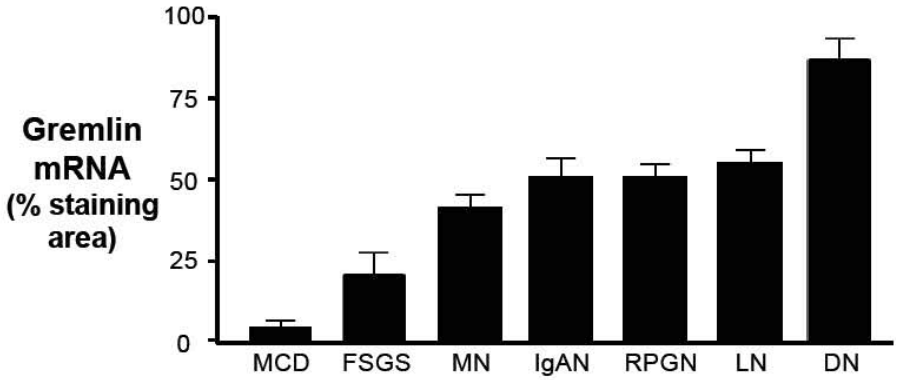

FIGURE 1. **Gremlin mRNA expression in human glomerulopathies.** Renal biopsies from 125 patients with glomerular diseases were examined, 12 with minimal change disease (MCD), 17 focal segmental glomerulosclerosis (FSGS), 33 membranous nephropathy (MN), 10 IgA nephropathy (IgAN), 32 rapidly progressive glomerulonephritis (RPGN), 8 lupus nephritis (LN) and 13 diabetic nephropathy (DN). The figure shows the quantification of gremlin mRNA levels, evaluated by *in situ* hybridization, expressed as % stained area vs. total area, as described in methods.

In MCD (no progressive nephropathy) there was no detection of Gremlin protein or mRNA (Figure 2A). In progressive nephropathies, Gremlin induction was mainly found in areas with interstitial fibrosis and it was detected in tubular and interstitial infiltrating cells. Gremlin mRNA was also expressed in the glomeruli; located in podocytes, mesangial, and parietal epithelial cells. As examples, a representative case of RPGN is shown in figure 2B and of MN in figure 3. In serial sections of this case of RPGN a clear co-localization between gremlin protein staining and mRNA can be seen in all renal structures (Figure 2B).

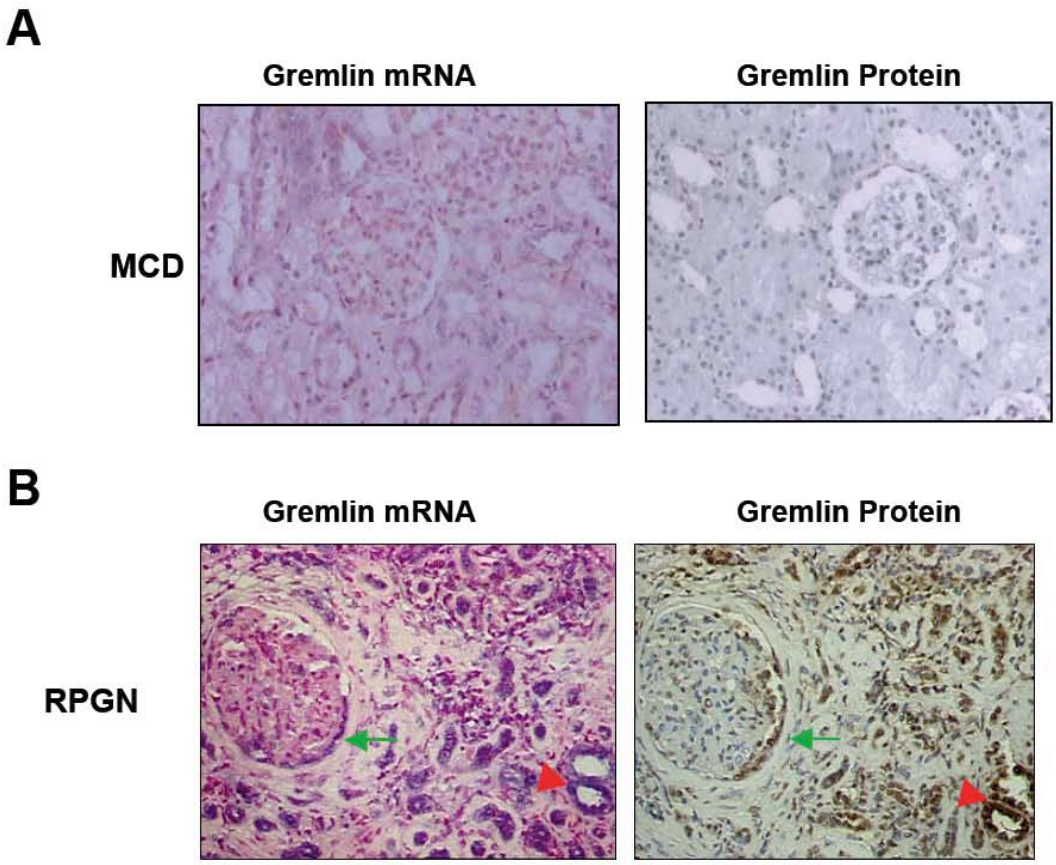

FIGURE 2. **Localization of gremlin gene and protein expression in non-progressive and progressive glomerulopathies.** **A.** In MCD, a non-progressive nephropathy, there was not detection of gremlin protein or mRNA. **B.** In RPGN, selected as an example of progressive nephropathy, gremlin mRNA was over-expressed in the glomeruli, located in podocytes, mesangial, and parietal epithelial cells. In tubulointerstitial areas, gremlin was mainly detected in tubuli and interstitial infiltrating cells. There was a clear co-localization between gremlin protein staining and mRNA, as indicated in epithelial parietal cells (arrow) and tubular cells (arrow head) of serial sections. Both figures (A and B) show a representative case of at least 10 studied. *In situ* hybridization and immunohistochemistry was done in serial sections of each biopsy to evaluate gremlin mRNA and protein expression, respectively. Magnification X200.

TIF is characterized by accumulation of EMC mainly produced by activated fibroblasts. EMT is one of the potential mechanisms responsible of the origin of myofibroblasts.[15-17] For this reason, in progressive glomerulopathies we have evaluated whether Gremlin induction was associated to changes in EMT markers. In normal kidney, the mesenchymal protein α-SMA is only present in arterioles and the epithelial marker E-cadherin is found at the basolateral membrane of tubular cells (not shown). Serial sections of a sample of progressive MN revealed that there is no expression of E-cadherin in some tubuloepithelial cells that express Gremlin. We also observed a strong upregulation of α-SMA and Gremlin in the tubulointerstitial area (Figure 3). Similar findings were observed in other progressive nephropathies, including RPGN and DN (not shown).

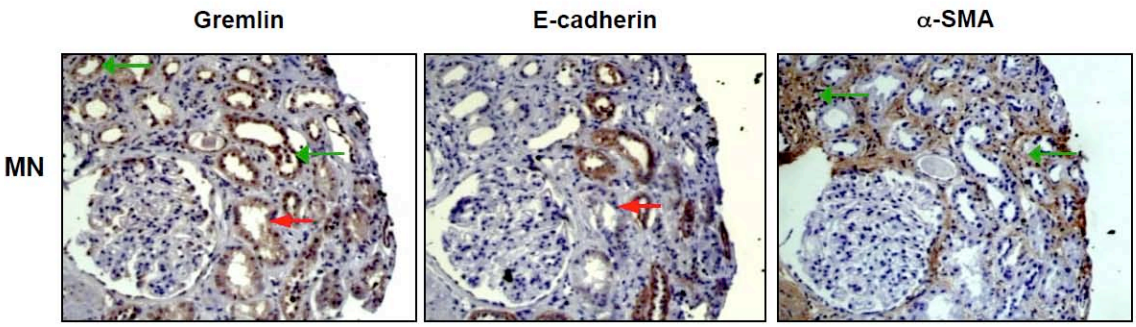

FIGURE 3. **Renal expression of gremlin and EMT markers in human biopsies of progressive glomerular diseases.** Figure shows a representative case of progressive MN. Induction of gremlin was found in glomeruli and tubulointerstitial cells. Tubular expression of E-cadherin was significantly decreased, and lost in some tubules. Gremlin was upregulated in many tubuli, including those without E-cadherin staining (red arrow). There was a strong induction of α-SMA in the surrounding interstitial area of gremlin positive cells (green arrow). The figure shows a representative case of 15 biopsies evaluated by immunohistochemistry. Magnification x 200.

Our findings in renal biopsies of patients with progressive glomerulopathies, show a relation between Gremlin induction, TIF and EMT changes, suggesting that Gremlin could participate in the regulation of human fibrosis and EMT in the kidney.

Induction of Gremlin is associated with TGF-β regulation and Smad activation in progressive nephropathies

By *in situ* hybridization of serial sections of human biopsies we evaluated the mRNA expression of Gremlin and TGF-β. In biopsies from patients with no progressive MN, a weak expression of Gremlin and TGF-β was found. In contrast, biopsies of progressive MN and DN showed strong co-expression of Gremlin and TGF-β mRNA mainly in cells from tubular epithelium (Figure 4).

Smad activation is a key event in TGF-β-induced fibrosis and EMT.[18-21] Smad activation was evaluated by Southwestern histochemistry, a technique that allows the detection of Smad-DNA binding activity in paraffin embedded tissues.[25,26] In biopsies from patients with Gremlin overexpression we observed active Smad complexes in podocytes, glomerular mesangial cells, and mainly in tubuloepithelial cells. As an example, figure 5 illustrates a representative case of serial sections of progressive MN showing colocalization of active Smad complexes and Gremlin upregulation in some tubulo-epithelial cells.

These data indicates that gremlin induction is associated with TGF-β/Smad activation in progressive nephropathies.

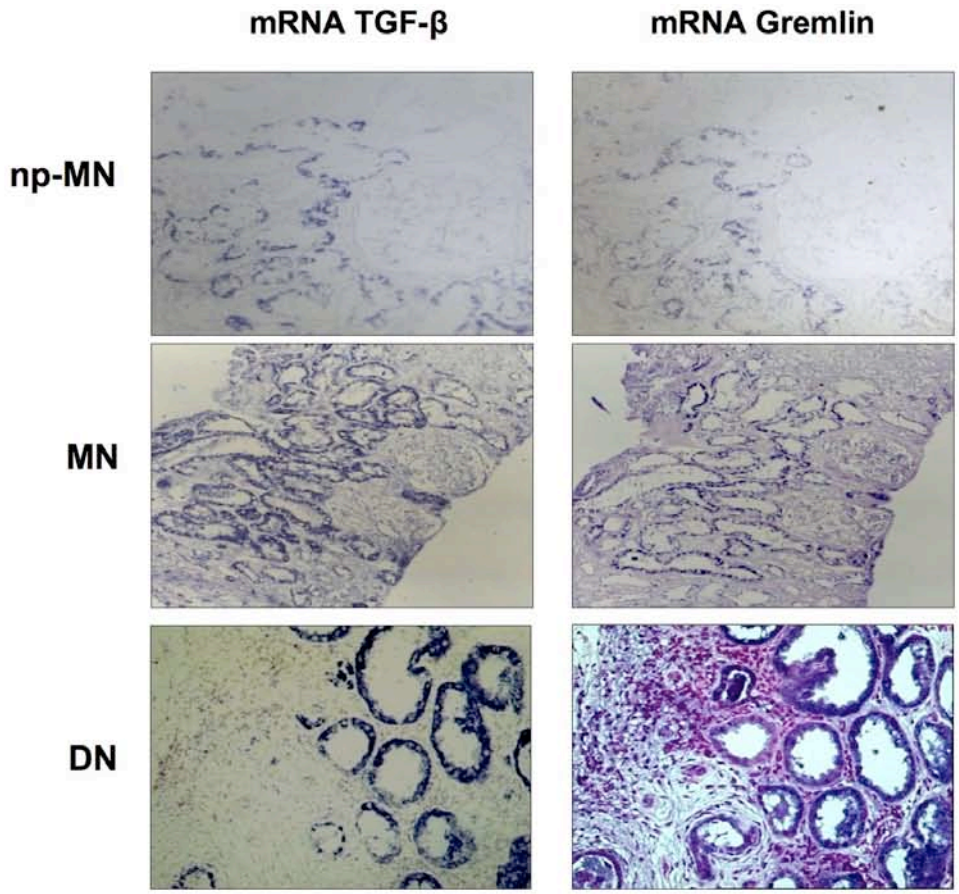

FIGURE 4. **Co-localization of gremlin and TGF-β mRNA in different glomerulopathies.** In serial sections of non-progressive MN, *in situ* hybridization shows weak gene expression of gremlin and TGF-β. In contrast, strong co-expression of gremlin and TGF-β mRNA was found in biopsies from patients with progressive MN and DN. Magnification X200.

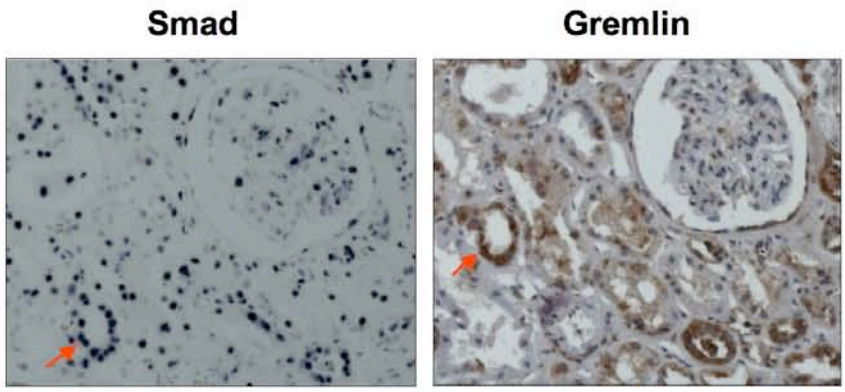

FIGURE 5. **Induction of gremlin was associated with Smad activation in human progressive glomerulopathies.** In serial sections of progressive MN, Smad activation was found in glomerular cells and mainly in tubuloepithelial cells. In some Smad-positive cells, gremlin overexpression was also observed (marked by an arrow). Magnification X200 Gremlin protein was evaluated by immunohistochemistry, and Smad activation by Southwestern histochemistry. The figures show a representative case of 10-15 biopsies studied.

DISCUSSION

The evaluation of a broad range of different human progressive glomerulopathies showed that Gremlin is associated with TIF and EMT changes, and the activation of TGF-β/Smad pathway. The present study suggests that Gremlin could participate in the EMT process through the regulation of TGF-β/Smad pathway.

Chronic progressive fibrosis of the kidney remains an unsolved challenge. The investigation of the mediators and mechanisms involved in renal fibrosis could lead to better diagnostic tools and novel therapeutics approaches. Previous studies suggest that Gremlin could be a key mediator of renal damage in diabetic nephropathy.[10-14] Now, we have studied 125 human biopsies of patients with progressive and non-progressive glomerular diseases. Our data show Gremlin overexpression in all progressive glomerulonephritis studied. In contrast, in non-progresive glomerular diseases (MCD) there is no Gremlin induction, as observed in normal kidneys. Gremlin is induced both in renal resident cells and in infiltrating inflammatory cells, mainly in areas of tubulointerstitial fibrosis. Moreover, Gremlin gene expression was correlated with the degree of TIF. These findings confirm the role of Gremlin in DN, and support a global pathogenic role of Gremlin in other human progressive glomerular diseases.

Emerging evidence indicates that a large proportion of interstitial fibroblasts are actually originated from tubular epithelial cells via EMT in injured kidney. [15-17] Our data showed changes of EMT markers in serial sections of biopsies from patients with progressive glomerulopathies in correlation with Gremlin overexpression in tubuloepithelial cells. However, these findings do not demonstrate that these epithelial cells do actually migrate into the interstitium, become myofibroblasts, and produce ECM components, and whether Gremlin is a mediator of this process. For this reason, future studies are needed to evaluate the direct effect of Gremlin in EMT.

TGF-β is known as the major promoter of EMT during embryogenesis, cancer progression and fibrosis. [27] In human biopsies of progressive nephropathies, a strong co-expression of Gremlin and TGF-β was found in correlation with fibrosis and changes in EMT markers. Previous studies have demonstrated that TGF-β induces gremlin expression in inflammatory and renal cells. [12,23,24] These findings suggest that Gremlin could be a mediator of TGF-β actions in the kidney. However, more studies are needed to evaluate the relationship between Gremlin and TGF-β in the kidney.

In conclusion, our results suggest that Gremlin could be a common pathogenic mediator in progressive glomerulopathies including DN, modulating the EMT process through regulation of the TGF-β/Smad signalling pathway.

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4. Gremlin está regulado por AngII y TGF-β, dos factores claves en la progresión del daño renal.

Inicialmente Gremlin fue identificado como uno de los genes del desarrollo que se induce *in vitro* al exponer células mesangiales humanas a altas concentraciones de glucosa, estrés mecánico cíclico y TGF-β.[87] Existen pocos estudios que evalúen los factores que regulan la expresión de Gremlin a nivel renal.

El siguiente objetivo de esta tesis ha sido evaluar si factores implicados en la progresión del daño renal como AngII y TGF-β son capaces de modular las acciones de Gremlin. En un estudio previo realizado por nuestro grupo observamos que la estimulación de células túbulo-epiteliales humanas en cultivo con TGF-β induce expresión de Gremlin.[18]

En este trabajo hemos observado un aumento significativo en la expresión de Gremlin ARNm y proteína en un modelo experimental de infusión sistémica de AngII en ratas. En células túbulo-epiteliales en cultivo observamos un aumento en la expresión de Gremlin, similar a la observada con TGF-β.

Estos hallazgos sugieren que AngII y TGF-β son capaces de modular la síntesis de Gremlin en el riñón, lo que refuerza el rol de Gremlin como citoquina profibrogénica.

ANGIOTENSIN II INDUCES GREMLIN EXPRESSION AND SYNTHESIS IN THE KIDNEY

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INTRODUCTION

The study of the role of Angiotensin II (AngII) in the progression of kidney disease is a very active field of research in biomedicine. Drugs that block the renin angiotensin system (RAS), as angiotensin converting enzyme inhibitors and type 1 receptor antagonists improve the clinical outcome of patients with chronic renal diseases.[1,2] Many studies including ours, have demonstrated activation of renal RAS and increased production of AngII in human and experimental kidney diseases.[3-5]

AngII regulates cell proliferation and hypertrophy, accumulation of extracellular matrix proteins and recruitment of proinflammatory cells in the kidney.[6,7] AngII is capable of inducing the synthesis of growth factors such as TGF-β and CTGF, cytokines such as IL-6, vasoactive peptides such as endothelin-1, and chemokines such as MCP-1, which mediate some AngII effects.[7,8]

Gremlin is a 184 amino acid protein that is located on the external cell surface and within the ER-Golgi compartments and is present in soluble or cell-associated forms.[9] It belongs to a family of bone morphogenetic proteins (BMPs) antagonists. Gremlin heterodimerizes with specific BMPs -2, -4 and -7, preventing the interactions with their specific receptors, this capacity is thought to be responsible for the critical role of Gremlin during the process of nephrogenesis.[10]

Gremlin is well known as a renal injury mediator in diabetic nephropathy.[11-14] Initially, Gremlin was identified as one of the developmental genes induced when human mesangial cells are exposed to high glucose *in vitro*.[15] There are few studies evaluating the factors involved in Gremlin regulation in the kidney. TGF-β1 was shown to induce Gremlin expression in human mesangial cells and in cultured human tubuloepithelial cells.[15,16] The aim of this study is to evaluate whether AngII could regulate Gremlin synthesis in the kidney.

MATERIAL AND METHODS

Experimental studies

Systemic infusion of AngII was done into male Wistar rats of 3 months of age (subcutaneous osmotic minipumps, Alza Corp., CA) at 100 ng/kg/min for 2 weeks (n=8 animals per group). A control group of saline-infused rats of the same age was also studied (n=8 animals). The kidneys were perfused *in situ* with cold saline before removal. One kidney was fixed, embedded in paraffin, and used for immunohistochemistry, and the other kidney was snap-frozen in liquid nitrogen for RNA studies. All experimental procedures were approved by the Animal Care

and Use Committee of our Institution, according to the guidelines for ethical care of the European Community.

Cell cultures

Human renal proximal tubuloepithelial cells (HK2 cell line) were grown in RPMI with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100U/ml penicillin, and 100μg/ml streptomycin, ITS and hydrocortisone in 5% CO_2 at 37°C. At 60-70% of confluence, cells were growth-arrested in serum-free medium for 24h before the experiments. AngII (10^{-7} mol/l) (Fluka) and human recombinant TGF-β1 (1 ng/ml) (Peprotech, Rocky Hill, NJ) were used as stimuli. Cell culture reagents were obtained from Life Technologies, Inc.

Protein and gene studies

Immunohistochemistry was done in paraffin-embedded renal sections. Renal sections of 4μg were deparaffinized, rehydrated, their endogenous peroxidase was blocked and incubated with primary antibody rabbit polyclonal anti-Gremlin (ABGENT, AP6133a, San Diego CA, USA), followed by incubation with the corresponding secondary antibody, and revealed by standard techniques.

Immunocytochemistry was performed in cells growing on coverslips, fixed in Merckofix (Merck) and permeabilized with 0.2% Triton-X100, incubated overnight with Gremlin antibody overnight al 4ºC, followed by fluorescein isothiocyanate-conjugated antibodies (FICT). Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined by a Leica DM-IRB confocal microscope.

Total RNA was isolated with Trizol (Gibco) and gene expression was analyzed by real-time PCR, performed on the ABI Prism 7500 sequence detection PCR system (Applied Biosystems, Foster City, CA) according to the manufacturer´s protocol. Assay ID used for Gremlin was Rn01509832_m1. For normalized data, different approaches were done using several housekeeping genes including GAPDH, Histone-3 and 18s ribosomal RNA expression (Rn99999916_m1 y Hs99999901_s). Each animal was evaluated independently by duplicate.

Statistical analysis

Results are expressed as n-fold increase over control as mean ± SEM. Differences between groups were assessed by one-way analysis of variance, followed by post-hoc Bonferroni

or Dunnett test, or Mann-Whitney test, as appropriate. P<0.05 was considered significant. Statistical analysis was conducted using the SPSS statistical software (version 11.0).

RESULTS

Systemic AngII infusion into rats increases Gremlin expression in the kidney

We have investigated whether AngII *in vivo* could induce renal Gremlin expression. In kidneys from rats infused with AngII for two weeks we observed a significant increase in Gremlin mRNA levels compared with control animals ***(FIGURE 1A)***. Protein expression was evaluated by immunohistochemistry. Gremlin was not expressed in normal control kidneys. In AngII-infused rats, elevated protein staining for Gremlin was found, mainly in the tubular epithelial cells ***(FIGURE 1B)***.

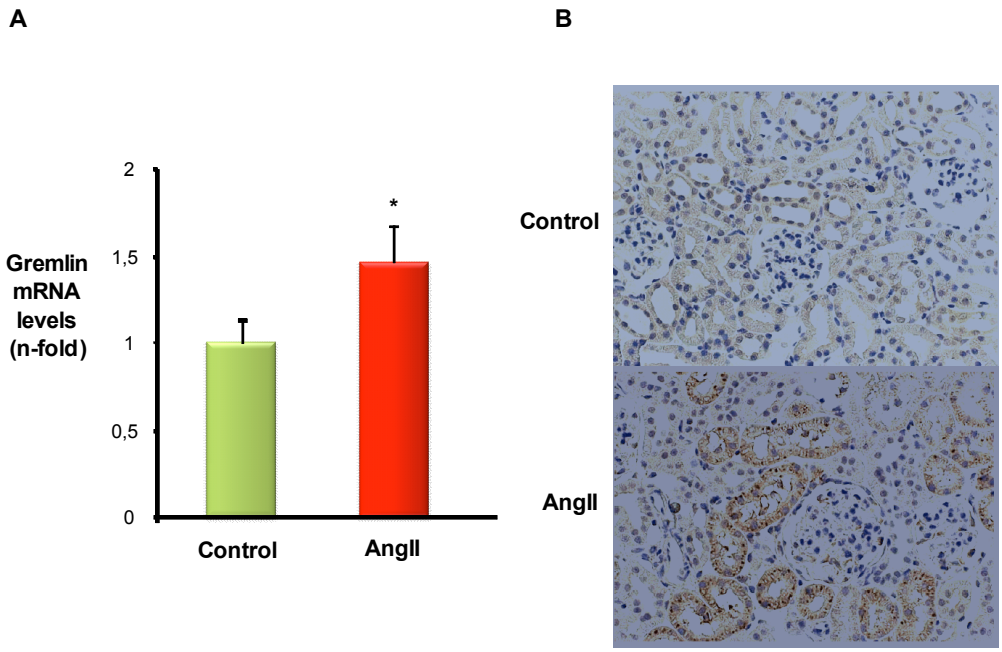

FIGURE 1. **Angiotensin II increases Gremlin gene expression and synthesis in the kidney.** Rats were infused with AngII (100 ng/kg/min) for 2 weeks and Gremlin mRNA and protein levels were evaluated by real time PCR **(A)** and immunohistochemistry **(B),** respectively. Panel **(A)** shows data of Gremlin gene expression, as mean ± s.e.m. of 8 animals per group. *p<0.05 vs control. Panel **(B)** shows a representative experiment of immunohistochemistry of a representative animal of each group.

AngII increases Gremlin expression in cultured human tubuloepithelial cells

To further demonstrate a direct effect of AngII in Gremlin synthesis *in vitro* studies were done. In cultured human tubuloepithelial cells, stimulation with 10^{-7} mol/l AngII or 1 ng/ml TGF-β for 48 hours induced Gremlin synthesis, observed by confocal microscopy **(*FIGURE 2*).**

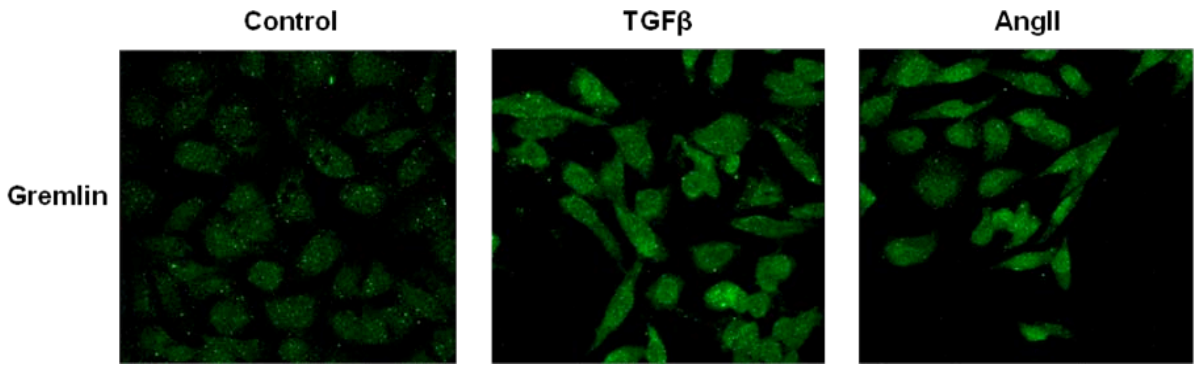

FIGURE 2. **AngII induces the synthesis of Gremlin in cultured human tubuloepithelial cells.** Cells were treated with 10^{-7} mol/l AngII or 1 ng/ml TGF-β for 48 hours. The evaluation of Gremlin was done by confocal microscopy with a fluorescein isothiocyanate-labeled secondary antibody. Figure shows a representative experiment of 3 done.

DISCUSSION

The investigation of the molecular mechanisms involved in renal fibrosis could lead to improve current clinical treatments for renal patients. Gremlin is a developmental gene that becomes activated during pathological changes in adult tissues.[17] Our *in vivo* experiments using a model of systemic infusion of AngII in rats showed a significant increase in Gremlin mRNA and protein expression levels in the kidney. AngII also upregulated Gremlin expression in cultured human tubuloepithelial cells, similar to that observed with TGF-β. These findings suggest that AngII is capable of modulating the synthesis of Gremlin in the kidney, thus strengthening the role of Gremlin as a profibrogenic cytokine.

TGF-β is well known as the most important renal profibrogenic cytokine.[18-20] TGF-β regulates Gremlin expression in several cultured renal cells.[13,16,21] Moreover, TGF-β mediates the stimulatory effect of high glucose on Gremlin expression in human mesangial cells.[15] Some authors suggest that Gremlin is induced by TGF-β in diabetic nephropathy, and contributes to progression of kidney injury.[11-14] In kidney biopsies from patients with diabetic nephropathy, we have also observed Gremlin expression, and this expression was most pronounced in areas of

interstitial fibrosis and was co-localized with TGF-β.[13] In these patients, local activation of RAS, including elevated production of AngII has been described.[5] Our data demonstrating that AngII could also regulates Gremlin show that several factors, besides TGF-β, can regulate Gremlin expression in the kidney.

Recent studies have demonstrated that Gremlin promotes cell migration, proliferation and apoptosis in VSMC. Such actions would be mediated by growth factors such as TGF-β, PDGF and AngII.[22,23] The induction of Gremlin by AngII in the kidney was observed after 2 weeks of infusion, at the same time of induction of changes in epithelial mesenchymal transition markers and the onset of renal fibrosis. We have recently observed that Gremlin induces epithelial mesenchymal transition in cultured human tubuloepithelial cells (unpublished data), however, future studies are needed to evaluate whether Gremlin is a mediator of AngII-induced renal fibrosis.

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5. Gremlin un nuevo mediador de la transición epitelio-mesenquimal.

La presencia de Gremlin asociada a marcadores de TEM observada en las biopsias de pacientes con nefropatías progresivas no demuestra que las células epiteliales efectivamente hayan sufrido una transformación fenotípica, ni que Gremlin sea el mediador del proceso. Por lo tanto, nuestro siguiente objetivo ha sido evaluar si Gremlin es capaz de inducir TEM *in vitro.*

La estimulación con Gremlin recombinante o la transfección con un vector de expresión de células túbulo-epiteliales en cultivo indujo cambios morfológicos hacia un fenotipo de fibroblasto, con disminución de marcadores epiteliales y sobre-expresión marcadores mesenquimales, además de un aumento en la expresión de fibronectina y MMP-9. Estos hallazgos se correlacionan con los eventos fundamentales del proceso de TEM.[57,62,151]

Dado que la activación de la vía TGF-β/Smad es clave en el proceso de TEM, [33,67,123] el último objetivo de esta tesis ha sido evaluar la participación de la vía TGF-β/Smad en las respuestas inducidas por Gremlin.

En células túbulo-epiteliales humanas en cultivo la estimulación con Gremlin recombinante aumentó la expresión del ARNm y proteína de TGF-β. Por otra parte, el bloqueo de TGF-β activo, utilizando un anticuerpo neutralizante, disminuyó la TEM inducida por Gremlin.

En células túbulo-epiteliales humanas la incubación con Gremlin indujo una rápida a activación de la ruta Smad, caracterizada por la fosforilación de Smad2 y Smad3, y la traslocación nuclear del trímero Smad 2/3 fosforilado y Smad4, observados a los 20 minutos de estimulación. En células túbulo-epiteliales humanas transfectadas con un vector de expresión de Gremlin observamos activación de Smad, confirmando los resultados obtenidos con la proteína recombinante. La co-transfección de Smad7 y el vector de expresión de Gremlin en células túbulo-epiteliales inhibió la activación de la ruta Smad y la TEM mediada por Gremlin.

Nuestros resultados muestran que en células túbulo-epiteliales humanas Gremlin induce transición epitelio-mesenquimal mediante la activación de la ruta TGF-β/Smad.

Gremlin, through the Smad signaling, induces epithelial mesenchymal transition in cultured tubuloepithelial cells

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Running title: **Gremlin and EMT**

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ABSTRACT

Epithelial mesenquimal transition (EMT) is one process involved in renal fibrosis. Our aim was to investigate whether gremlin, a BMP-7 antagonist, participates in EMT and its relation with the TGF-β/Smad pathway. Incubation of human tubuloepithelial cells with gremlin for 2 days caused a phenotype conversion into myofibroblasts-like cells, characterized by loss of the epithelial markers E-cadherin and cytokeratin and induction of mesenchymal markers (α-smooth muscle actin and vimentin). Transfection of HK2 cells with a gremlin expression vector also caused EMT phenotypic changes. We have further evaluate the involvement of TGF-β/Smad pathway in Gremlin-induced responses. Stimulation of human tubular epithelial cells (HK2 cell line) with gremlin increased TGF-β mRNA and protein release. The blockade of TGF-β, by a neutralizing antibody against active TGF-β, diminished gremlin-induced EMT changes. Gremlin caused a rapid activation of Smad signaling (Smad2/3 phosphorylation and nuclear translocation) in tubuloepithelial cells. Smad7 overexpression, which blocks Smad2/3 activation, diminished Smad-dependent gene transcription and EMT changes in gremlin-transfected tubuloepithelial cells. In conclusion, we propose that gremlin is involved in renal fibrosis by inducing EMT through TGF-β/Smad signaling.

INTRODUCTION

Gremlin, a member of cysteine knot superfamily, is a protein of 184 amino acid, glycosylated, phosphorylated and secreted, present both on the external cell surface and within the endoplasmic reticulum-Golgi compartments, which has soluble and cell-associated forms (1,2). Gremlin belongs to a family of bone morphogenetic proteins (BMPs) antagonists and is highly conserved during evolution (3). Gremlin heterodimerizes with BMPs -2, -4 and -7, preventing their interactions with specific receptors and this capacity is thought to be responsible for the critical role of gremlin during the process of nephrogenesis, fibrosis, and cancer (3-7). However, BMP-independent mechanisms may mediate several gremlin intracellular actions, such its ability to suppress tumorigenesis and stimulates endothelial-cell migration (8,9). Taken together these observations indicate that gremlin may exert multiple functions in different physiopathologic conditions via BMP-dependent and BMP-independent mechanisms.

Gremlin, also called down-regulated by mos (Drm) and initially known as induced in high glucose-2 (IHG-2), was identified as one of the developmental genes induced in cultured human mesangial cells exposed to high glucose (10). Gremlin is a well-known mediator of renal injury in diabetic nephropathy (DN) (11-13). Transforming growth factor-β1 (TGF-β1) was shown to induce gremlin expression and to mediate the effect of high glucose on this gene in human mesangial cells *in vitro* (10). A recent study using a streptozotocin-induced model of type 1 diabetes in knockout mice heterozygous for grem1 gene showed that several fibrotic-related proteins, such as fibronectin and connective tissue growth factor, were attenuated in grem1(+/-) mice compared with in wild-type controls (14).

Irrespective of the underlying cause, chronic kidney disease is linked with the development of tubulointerstitial fibrosis (TIF), characterized by accumulation of extracellular matrix (ECM). The key cellular mediator of fibrosis is the myofibroblast. In addition to activated local tissue fibroblasts, myofibroblasts can originate from circulating mesenchymal progenitors or from epithelial cells in a process known as epithelial-mesenchymal transition (EMT) (15-17). There is a change in morphology from the cobblestone-like cell morphology typical of an epithelial phenotype to the elongated, fusiform cell sheet characteristic of fibroblasts. The epithelial cells lose polarity and intercellular adhesion and gain mesenchymal properties including motility. During this process epithelial markers (E-cadherin, cytokeratin) are lost and mesenchymal markers, such as vimentin and α-smooth muscle actin (α-SMA), increase (15-17).

TGF-β is a key player in EMT, being the Smad proteins the main signalling pathway involved in this process (18-21). Because of its pleiotropic actions, TGF-β blockade is not an ideal therapeutic tool and novel targets are needed. Among them, gremlin may be an interesting candidate in progressive renal diseases (22). Recent *in vitro* studies developed by our group have shown gremlin induction by TGF-β in cultured proximal tubulo-epithelial cells and monocytes (23,24). We also reported the presence of gremlin in cellular crescents of pauci-inmune glomerulonephritis and in tubulointerstitium of chronic allograft nephropathy, correlated with the degree of TIF and associated with evidence of EMT-related changes (23,24).

The aim of this work was to evaluate whether gremlin could directly modulate EMT in cultured tubuloepithelial cells, evaluating the involvement of the TGF-β/Smad pathway in this process.

RESULTS

Gremlin causes EMT in cultured human tubuloepithelial cells

Incubation of cultured human tubuloepithelial cells (HK-2 cell line) with gremlin (human -hGrem1- and murine –mGremlin- recombinant proteins) for 48 h caused phenotypic EMT changes. The transformed cells lost their typical cobblestone pattern of an epithelial monolayer and displayed a spindle-shaped, fibroblast-like morphology, assessed by contrast phase microscopy (Figure 1A). In unstimulated tubuloepithelial cells, E-cadherin is expressed at the cellular membrane and there is no staining for the mesenchymal markers vimentin or α-SMA. After treatment with murine gremlin for 48 h E-cadherin was internalized and finally disappears. Gremlin stimulation also caused induction of vimentin and α-SMA filaments in the cytoplasm (Figure 1B, confocal microscopy). By western blot we observed that recombinant gremlin (murine and human) induced the loss of the epithelial marker cytokeratin and *de novo* expression of vimentin after 48 h (Figure 1C). The upregulation of ECM proteins, such as fibronectin and several types of collagens, and of metalloproteases 2 and 9, both implicated in the degradation of basement membranes, are also pathogenic elements in the process of renal fibrosis and EMT (15-17). In HK2, Gremlin increased mRNA levels of MMP-9 after 24 h (Figure 2A), and soluble fibronectin after 48 h of stimulation (Figure 2B). These data indicate that gremlin is capable of induce EMT and fibrosis in cultured human tubuloepithelial cells.

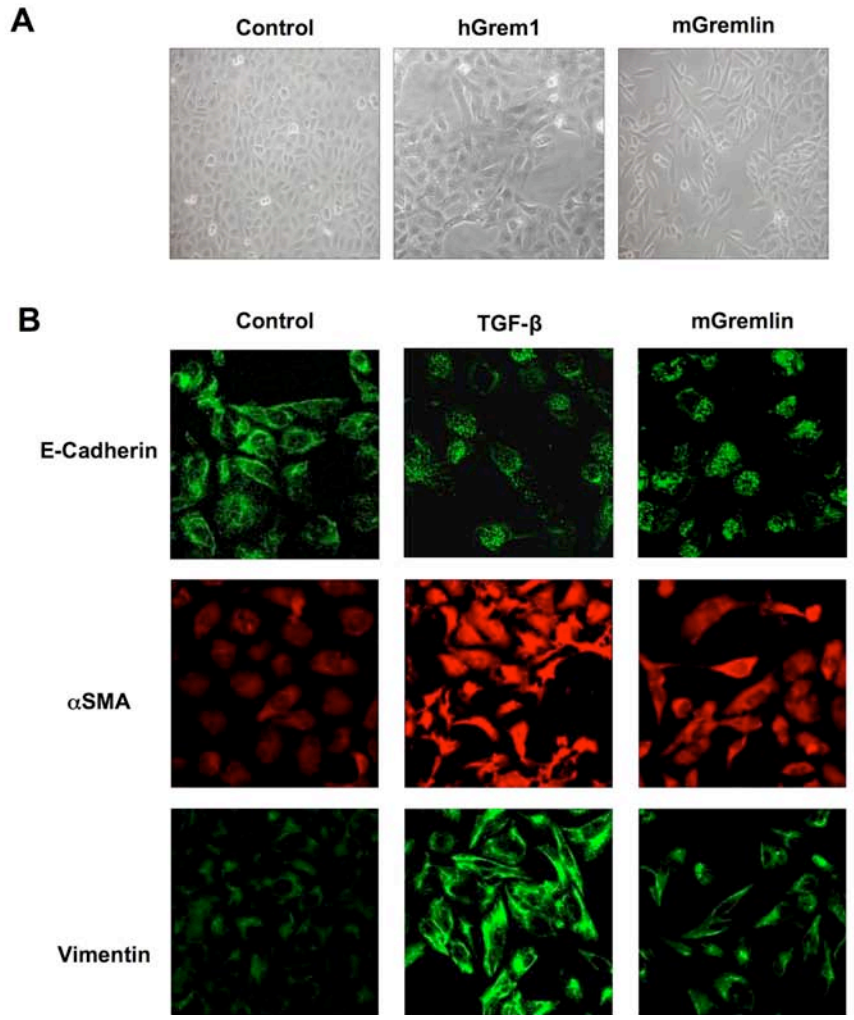

FIGURE 1. Gremlin causes EMT in human tubuloepithelial cells. A. HK2 cells were stimulated with recombinant human gremlin (hGrem1, 1μg/ml), murine gremlin (mGremlin; 5μg/ml) or 10 ng/ml of TGF-β1 for 48 hours. Phase-contrast images were taken at 200x . magnification. **B. Gremlin induces the loss of E-cadherin and de novo expression of vimentin and α-SMA.** The E-cadherin, vimentin and α-SMA were detected by an indirect immunostaining using a mouse FITC-labeled secondary antibody. Figure shows a representative picture of 4 confocal microscopy experiments **C. Quantification of EMT changes.** Figure shows a representative western blot (bottom) of total protein expression of vimentin and cytokeratin. Data (top) were obtained from densitometric analysis and expressed as protein/G3PDH ratio as n-fold over control of mean±SEM of 3 independent experiments. *p<0.05 vs. control.

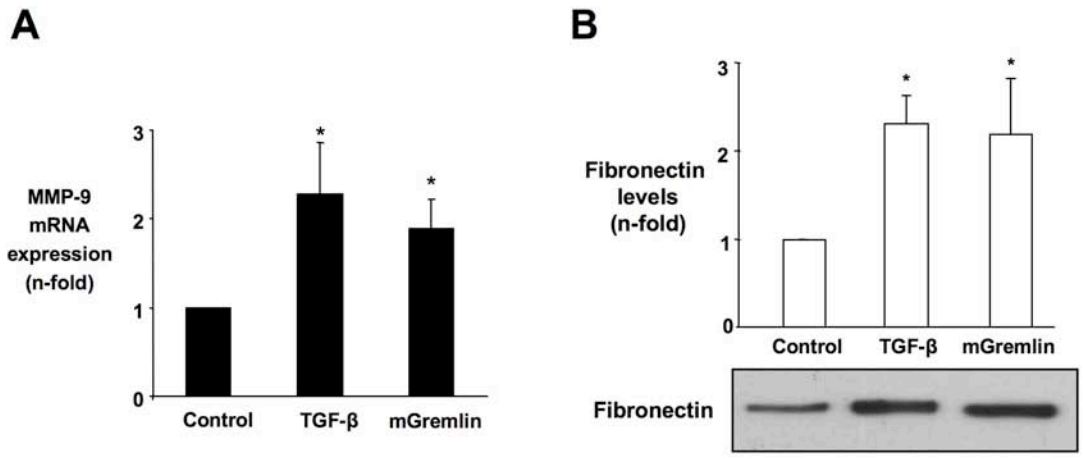

FIGURE 2. Gremlin induces release of ECM proteins. **A.** Total cell RNA was isolated to assess mRNA levels of MMP-9 by real-time PCR. **B.** Fibronectin levels were evaluated in cell supernatants after 48 hours of gremlin stimulation. Bottom panel shows a representative Western blot and data (top) of total protein levels as mean±SEM of 3 independent experiments. *p<0.05 vs. control.

Gremlin increases TGF-β in cultured human tubuloepithelial cells

Incubation of cultured tubuloepithelial cells with gremlin for 24 h induced a significant upregulation of TGF-β mRNA levels (Real Time PCR, Figure 3A). After 48 h of stimulation, active TGF-β protein was also increased in the supernatants of stimulated cells (ELISA, Figure 3B).

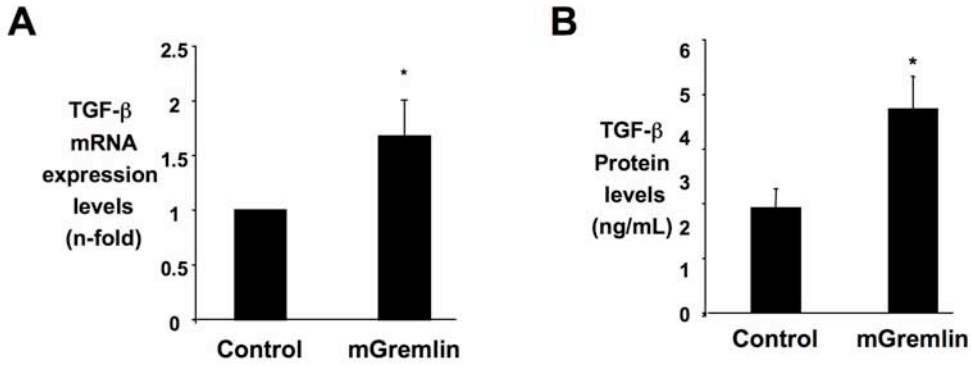

FIGURE 3. Gremlin induces TGF-β1 in human tubuloepithelial cells. HK2 cells were stimulated with murine gremlin (mGremlin; 5μg/ml) for 24 (mRNA) and 48 (protein) hours in serum-free medium. Total cell RNA was then isolated to assess mRNA levels of TGF-β1 **(A)** by real-time PCR **(B).** TGF-β1 was measured in the cell-conditioned medium by a specific ELISA. Data are expressed as mean ± SEM of 3 (A) and 6 (B) independent experiments. *p<0.05 vs. control.

Role of endogenous TGF-β on gremlin induced EMT

We next blocked TGF-β at the time of HK-2 stimulation with gremlin by adding a neutralizing antibody against active TGF-β, which has proven to block angiotensin II-induced ECM production and EMT (26). This blockade antagonized several EMT-related changes induced by gremlin, as shown by immunofluorescence (Figure 4). These data suggest that TGF-β is a mediator of EMT changes induced by gremlin in tubuloepithelial cells.

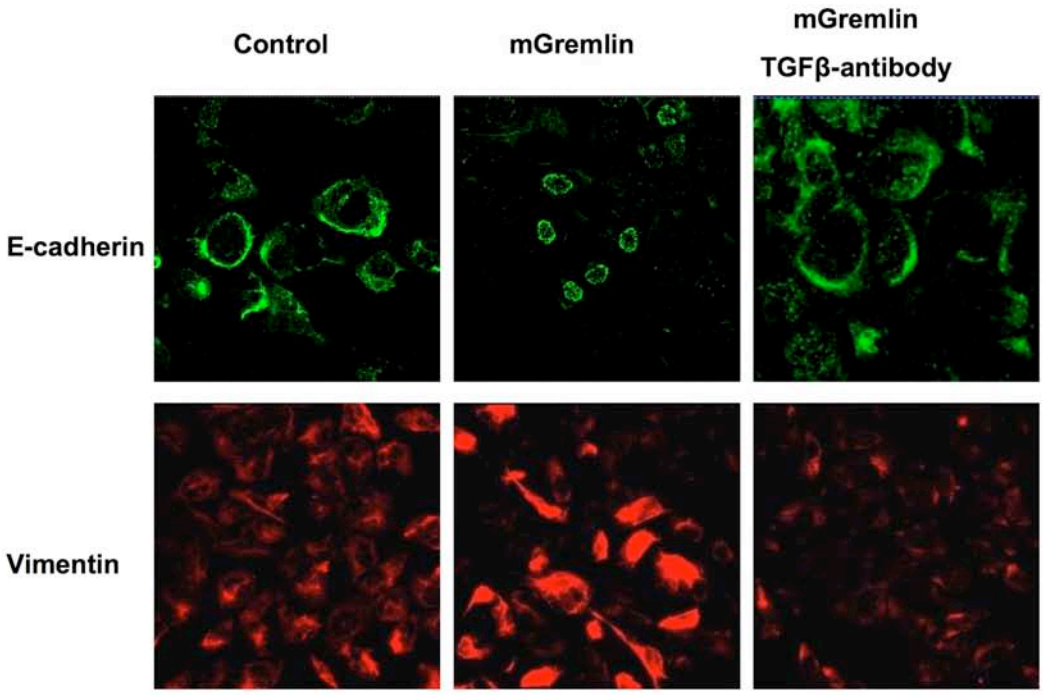

FIGURE 4. TGF-β acts as a mediator of Gremlin–induced EMT. TGF-β was blocked or not (control) by pre-treatment of cells for 1 h with an anti-TGF-β neutralizing antibody. Cells were then stimulated with 5μg/ml murine gremlin (mGrem1) for 48 hours. Detection of E-cadherin and vimentin was performed by indirect immunofluorescence using FITC-labeled secondary IgG and confocal microscopy. This represents the results of 3 independent observations.

Gremlin activates Smad pathway in human cultured tubuloepithelial cells

Receptor mediated activation of Smad proteins (R-Smads 2 and 3) occurs by direct C-terminal phosphorylation. Smad2/3 then form trimers with Smad4, and translocate into the nucleus, where they associate and cooperate with DNA binding transcription factors to activate or repress target gene transcription (21). In cultured HK2 cells, stimulation with recombinant gremlin for 20 min increased total phosphorylation levels of Smad2 and Smad3 and induced the nuclear translocation of phosphorylated-Smad2/3 (p-Smad) and Smad4 (figure 5).

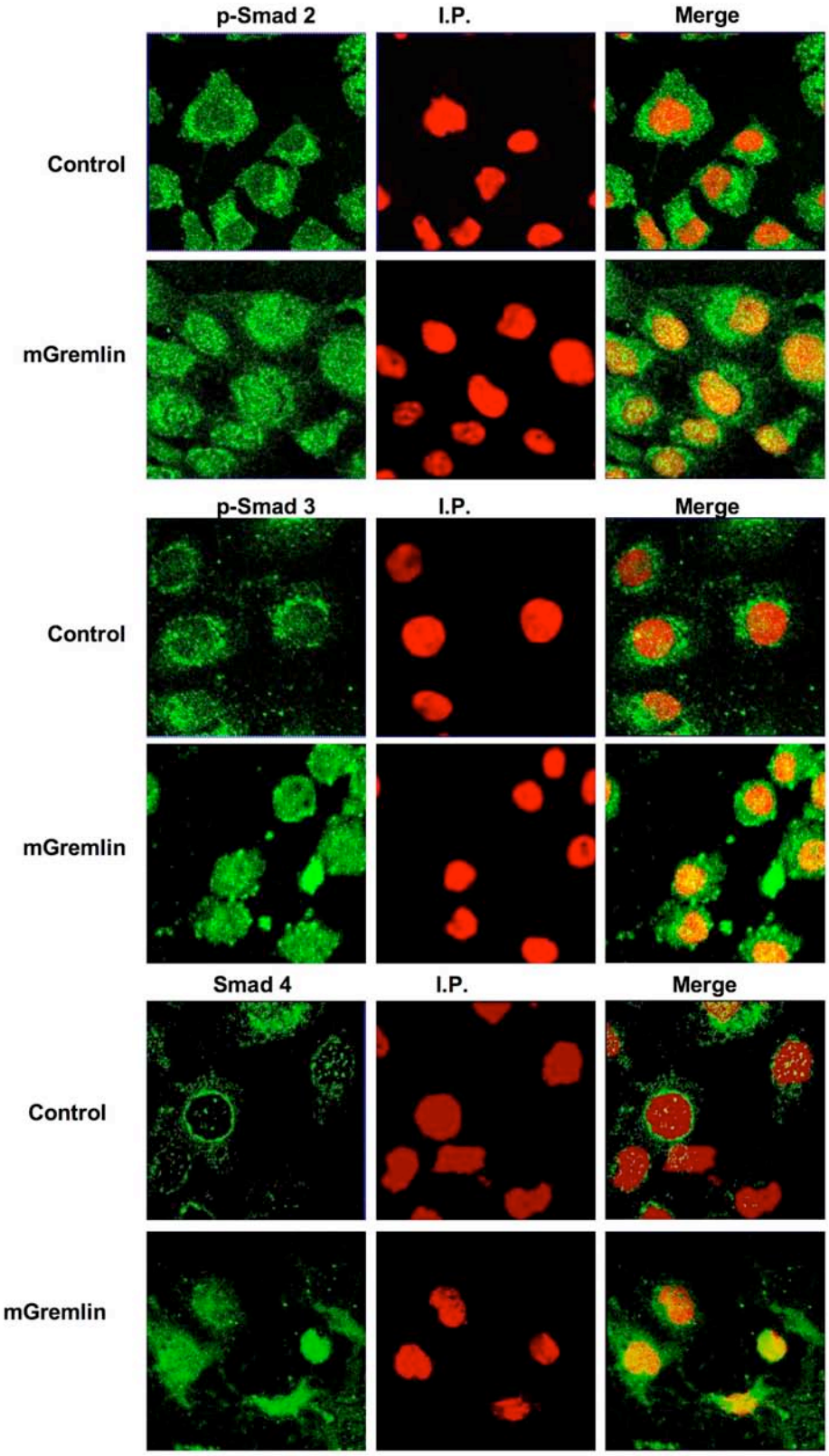

FIGURE 5. Gremlin induces a rapid activation of the Smad pathway in cultured human tubuloepithelial cells. Cells were treated with 5μg/ml mGremlin for 20 min. Smad4 and p-Smad2/3 were evaluated by confocal microscopy with FITC-labeled secondary antibodies (green staining). Nuclei were stained with propidium iodide (in red). In the merge, the yellow staining indicates nuclear localization of Smad proteins. The results are representative of 3 independent experiments.

To confirm that gremlin activates the Smad pathway, cells were transfected with a gremlin expression vector (GREM-GFP). By confocal microscopy we observed that after 24 h positive gremlin transfected cells (green staining) presented nuclear p-Smad2 and p-Smad3 immunostaining (red nuclear staining). This can be seen more clearly in the overlay image (positive white nuclear staining). In control and non-transfected cells, there is no nuclear p-Smad2 and p-Smad3, as seen by blue staining in the nucleus (figure 6).

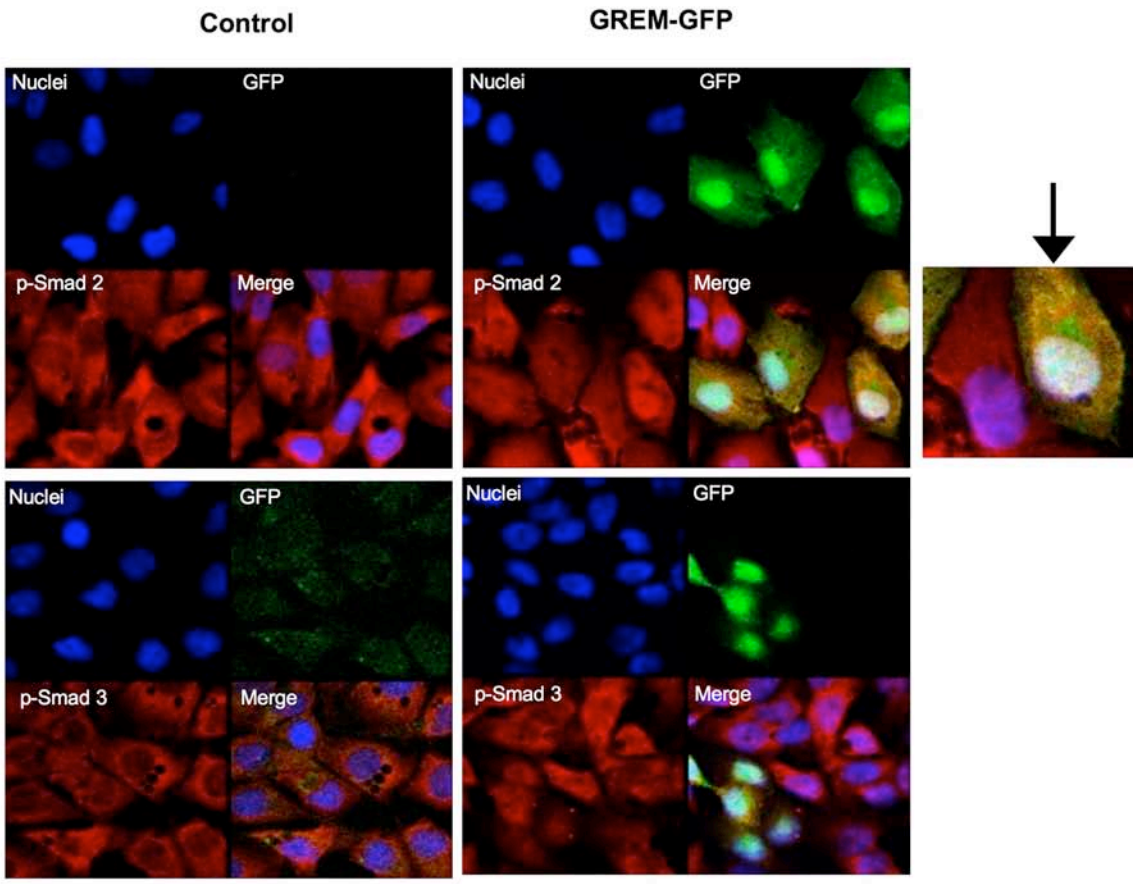

FIGURE 6. A. Gremlin overexpression activates the Smad pathway. HK2 cells were transiently transfected a gremlin expression vector (GREM-GFP) and phosphorylated-Smads (p-Smad2/3) were evaluated after 24 hours by confocal microscopy. The nucleus were stained using 4´,6-diamino-2-phenylindole dihydrochloride (DAPI) (blue) and a Alexa-633 secondary IgG (red). Only in gremlin transfected cells (green staining by GFP), p-Smad2 was found in the nuclei. In the insert, the overlay image shows p-Smad2 nuclear localization in gremlin positive cells (arrow), that yielded a white tone in the nucleus, while in non-transfected cells blue nuclear staining was observed. Similar results were observed in Smad3 phosphorylation studies.

To investigate whether gremlin regulates Smad-mediated gene expression, cells were co-transfected with a gremlin expression vector (GREM-GFP) and a luciferase Smad reporter plasmid. Gremlin transfected cells expressed higher Smad-dependent luciferase activity than controls after 24 h (figure 7). In parallel experiments, we observed that recombinant gremlin also increased Smad-mediated gene transcription (not shown).

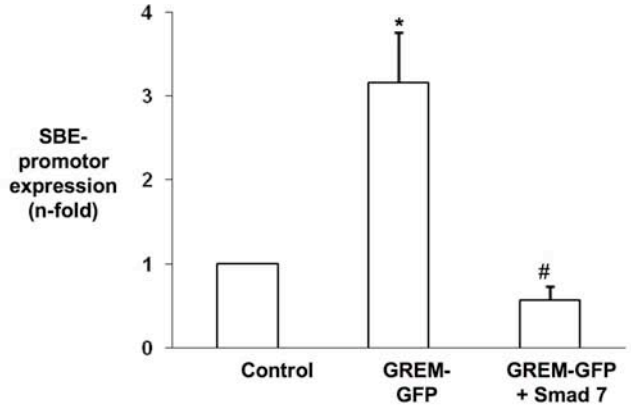

FIGURE 7. Gremlin induces Smad-dependent gene transcription. HK2 cells were transfected with GREM-GFP, Smad/luc promoter and TK-renilla for 24 hours. In some points, cells were contransfected with Smad7. Then, luciferase/renilla activity was measured. Data of mean±SEM of 5 experiments done. *p<0.05 vs. control. # p<0.05 vs. GREM-GFP.

Gremlin-induced EMT is mediated by Smad activation.

To further demonstrate the involvement of Smad pathway in gremlin-induced responses, a Smad7 expression vector, that inhibits TGF-β/Smad-mediated transcriptional effects by interfering with receptor-mediated activation of R-Smad, was used (21,27). The Smad promoter activity was significantly lower in HK2 cells co-transfected with GREM-GFP and Smad7 expression vectors than in gremlin-transfected cells (figure 7), supporting the involvement of Smad2/3 pathway in gremlin-induced cellular responses.

In HK2 cells transfected with GREM-GFP for 48 h, EMT-related phenotypic changes were observed by confocal microscopy. Gremlin-expressing cells (GREM-GFP positive; green staining) presented reduction of cytokeratin staining and induction of α-SMA (yellow staining and fibroblast-like morphology) (figure 8). In the same preparation, gremlin non-expressing cells presented epithelial morphology and EMT markers similar to those observed in control cells. Co-transfection of gremlin and Smad7 diminished these EMT changes (figure 8). These data suggest that gremlin regulates EMT through the Smad pathway.

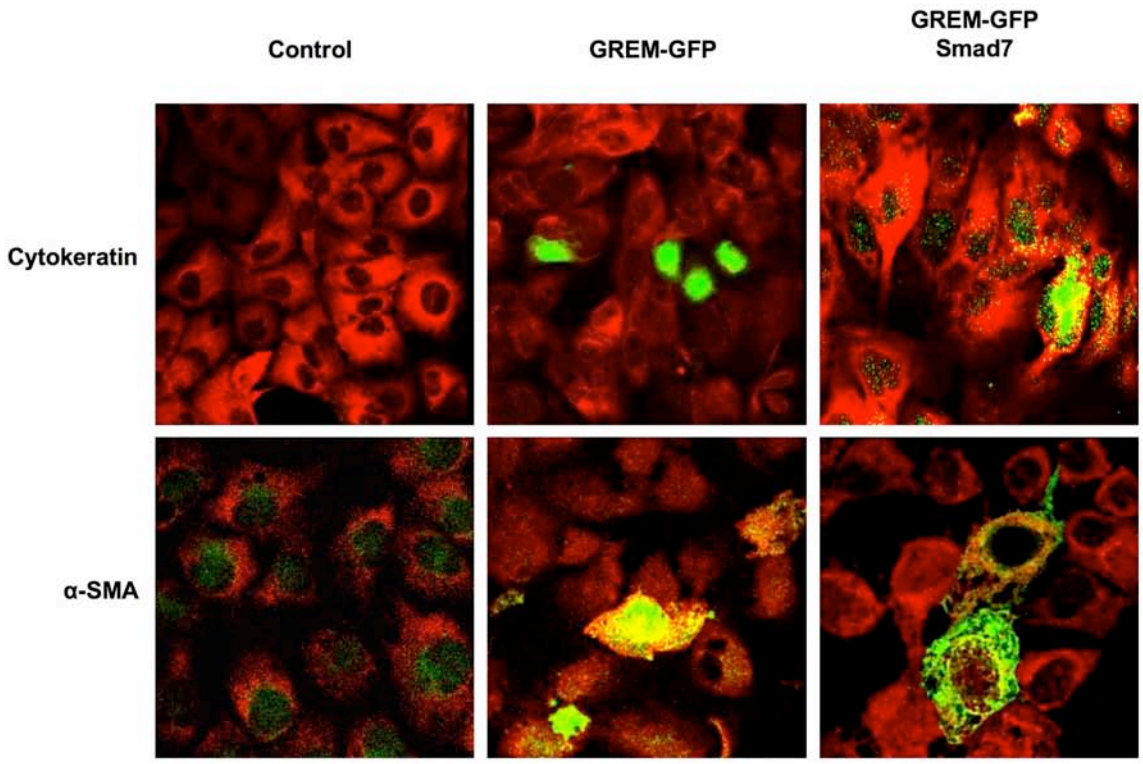

FIGURE 8. Gremlin-induced EMT via Smad pathway. HK2 cells were transiently transfected a gremlin expression vector (GREM-GFP) and/or Smad7 expression vector. EMT markers were evaluated after 48 hours. Gremlin transfected cells express GFP (green staining). Confocal microscopy analysis of cytokeratin and α-SMA immunofluorescence were performed using specific primary antibodies and an Alexa-633 secondary IgG (red staining). Figures show a representative experiment of 3 done.

DISCUSSION

Our in vitro studies in cultured tubuloepithelial cells show that gremlin participates in the EMT process, via TGF-β/Smad pathway. These data suggest that gremlin could be a mediator of renal fibrosis.

Chronic progressive fibrosis of the kidney remains an unsolved challenge. The investigation of the mediators and mechanisms involved in renal fibrosis could lead to better diagnostic tools and novel therapeutics approaches. Previous studies suggest that gremlin participates in the pathogenesis of diabetic nephropathy (10-14).

In cultured human tubuloepithelial cells, the stimulation with recombinant gremlin or transfection with a gremlin expression vector elicited morphological changes to a fibroblast phenotype, downregulation of cytokeratin and E-cadherin, and upregulation of α-SMA and

vimentin. The EMT process occurring after development is linked to generation of matrix-producing fibroblasts. Our study also showed that gremlin upregulated fibronectin and MMP-9 in HK2 cells. All these findings did correlate with the key steps of EMT: cells lose their epithelial characteristics, including their polarity and cell to cell contact, and acquire a migratory behavior (15-17).

TGF-β is known as the major promoter of EMT during embryogenesis, cancer progression and fibrosis (28). Previous studies have demonstrated that TGF-β induces gremlin expression in inflammatory and renal cells (12,23,24). Here we show that in cultured human tubuloepithelial cells, gremlin is capable of induce TGF-β mRNA expression and protein release, while TGF-β acts as a downstream mediator of gremlin-induced EMT. All these findings reveal the complex relationship between gremlin and TGF-β in the kidney, disclosing a positive feed-back loop connection between them in promoting EMT and fibrosis.

Few studies have investigated the intracellular BMPs-independent mechanisms elicited by gremlin. Although there is no described cell-surface receptor, several data support the capacity of gremlin to exert direct cellular effects. In tumor-derived cell lines gremlin can suppress tumorigenesis involving up-regulation of p21 (Cip1) and phosphorylated p42/44 MAPK (29). Gremlin has been shown to bind Slit proteins and to inhibit monocyte chemotaxis (30). In endothelial cells gremlin increased the phosphorylation of tyrosine containing proteins, including paxillin, focal adhesion kinase, and the mitogen activated protein kinase (MAPK) extracellular signal-regulated protein kinase1/2 (ERK1/2), stimulating cell migration and invasion with possible implications in angiogenesis (9).

In the kidney the Smad route regulates EMT induced by key factors involved in renal fibrosis, such as TGF-β and Angiotensin II (18,19,25,26,31). Our study reveals that in cultured human tubuloepithelial cells gremlin induces a rapid activation of the Smad pathway, characterized by phosphorylation of Smad2 and Smad3 and nuclear translocation of Smad2/3 and Smad4 after 20 min. The activation of Smad2/3 pathway was confirmed in gremlin transfected cells. In tubuloepithelial cells Smad7 overexpression inhibited gremlin-mediated Smad activation and EMT changes. Our study demonstrates for the first time involvement of the Smad pathway in gremlin responses in renal tubular cells. In cultured optic nerve head astrocytes and lamina cribrosa cells recombinant gremlin stimulates ECM production through the activation of TGF-β receptor and Smad3 phosphorylation, suggesting a role for gremlin in glaucoma (32). Gremlin mRNA levels were up-regulated in the asbestos-exposed mouse lungs, which is in agreement with the human idiopathic

pulmonary fibrosis biopsy data. Accordingly, analyses of cultured human bronchial epithelial cells indicated that asbestos-induced gremlin expression could be prevented by inhibitors of the TGF-β receptor and also by inhibitors of the MAPK/ERK pathway (5). All these recent data suggest that gremlin could be an important promoter of fibrosis in different pathologies.

In conclusion, our results show that gremlin could mediate in the process of EMT through regulation of TGF-β/Smad signalling pathway.

METHODS

Cell cultures

Human renal proximal tubuloepithelial cells (HK2 cell line) were grown in RPMI with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 100U/ml penicillin, and 100μg/ml streptomycin, ITS and hydrocortisone in 5% CO_2 at 37°C. At 60-70% of confluence, cells were growth-arrested in serum-free medium for 24h before the experiments. Recombinant gremlin (murine and human) proteins were from R&D, human recombinant TGF-β1 (10ng/ml) (Peprotech, Rocky Hill, NJ). Cell culture reagents were obtained from Life Technologies, Inc. The TGF-β inhibitor used was an anti-TGFβ neutralizing antibody that recognizes bovine, mouse and human TGF-β1 and β2 isoforms (1μg/ml) (R&D).

Transfection, DNA Constructs and promoter studies

HK2 cells were transiently transfected for 24-48 h with FuGENE (Roche), pCDNA3-Gremlin-myc-IRES2-eGFP plasmid (GREM-GFP) and/or PcDNA3-FLAG-Smad7 expression vector (kindly donated by Dr. Massagué, Memorial Sloan-Keternig Cancer Center, USA) or empty vector (pcDNA3B). The GREM-GFP was generated as follows: human gremlin cDNA purchased in the plasmid pCR4 TOPO from the Mammalian Gene Collection (NIH). PCR was performed with primers that included a c-myc tag in the 3´portion of gremlin and new NotI restriction site. The IRES-eGFP sequence was obtained by PCR with specific primers using a plasmid containing MecP2 IRES-eGFP kindly donated by Dr. Juan Young as template. Gremlin-myc and IRES-eGFP were subcloned into a modified pCDNA3 vector. Gremlin-transfected cells expressed GFP, myc

and gremlin protein, observed by confocal microscopy using a specific anti-gremlin antibody (not shown). Controls to confirm the correct sequence were also done. To demonstrate Smad7 transfection efficacy an anti-FLAG antibody was used (not shown). Smad-dependent promoter activation was evaluated by transfection of Smad/luc (kindly donated by Dr. Volgestein, Baltimore, USA) and TK-renilla as internal control, as described (27)

Protein studies

Cell protein extracts (10-50µg/lane) obtained in lysis buffer were separated on 8-12% polyacrylamide-SDS gels under reducing conditions. Samples were then transferred onto nitrocellulose membranes (Bio-Rad), blocked with 5% nonfat dry milk; in 50mM Tris-HCl, pH 7.5, 150mM NaCl with 0.05% Tween-20, and incubated overnight at 4ºC with the primary antibodies and subsequently incubated with peroxidase-conjugated IgG (Amersham), and developed by ECL chemiluminiscence .

Cells grown on coverslips were stimulated with the agonists, and then fixed in Merckofix (Merck) and permeabilized with 0.2% Triton-X100 for 1 min. After blocking with 10% BSA and 10% serum for 1 h, they were incubated with several primary antibodies overnight at 4ºC, followed by a fluorescein isothiocyanate (FITC)-conjugated secondary antibody [1/200], or AlexaFluor® 633 [1/300] (Invitrogen) for 1 h. Nuclei were stained with 1µg/ml propidium iodide or 4′,6-Diamidino-2-phenyindole, dilactate (DAPI) (Sigma-Aldrich). Absence of primary antibody was used as negative control. Samples were mounted in Mowiol 40-88 (Sigma-Aldrich) and examined by a Leica DM-IRB confocal microscope.

Antibodies employed were: α-SMA (DAKO) (IF: 1/200), Vimentin (BD Pharmingen) (IF: 1/200; WB: 1/10.000), E-cadherin (R&D) (IF: 1/200), Pan-cytokeratin (Sigma-Aldrich) (IF: 1/200; WB: 1/2500), Fibronectin (Chemicon) (WB: 1/1000); p-Smad2 (Cell Signaling) (IF:1/300; WB: 1/2500), p-Smad3 (Abcam) (IF: 1/300; WB:1/2500), and Smad4 (Sta. Cruz) (IF: 1/300). The efficacy of protein loading and transfer to membranes was assessed by GAPDH (Chemicon) (WB: 1/5000).

Gene studies

Total RNA was isolated from cells with Trizol (Invitrogen). cDNA was synthesized using the High capacity cDNA Archive Kit (Applied) using 2µg of total RNA primed with random hexamer primers, following the manufacturer's instructions. Real time PCR was performed using human fluorogenic TaqMan MGB probes and primers designed by Assay-on-DemandTM gene expression products (Applied): MMP-9: Hs00234579_m1, TGF-1β: Hs99999918_m1. Data were normalized to 18S eukaryotic ribosomal RNA: 4210893E (VIC). The mRNA copy numbers were calculated for each sample by the instrument software using Ct value ("arithmetic fit point analysis for the lightcycler"). Results were expressed in copy numbers, calculated relative to unstimulated cells after normalization against 18S.

Statistical analysis

In situ hybridization was quantified by image analysis using a KZ 300 imaging system 3.0 (Zeiss). The staining score is expressed as % stained area, and it was calculated as the ratio of stained area vs. the total field area. For each sample, the mean staining area was obtained by analysis of 20 different fields (x200). In all cases, evaluations were performed by two independent observers in a blinded fashion and the mean score value calculated for each sample.

Tubulointerstitial cell infiltration and interstitial fibrosis (TIF) was classified into four groups according to their extent and the presence of tubular atrophy, and scored between 0-100 % following this criteria: a) normal, b) involvement up to 25% of the cortex, c) involvement of 26 to 50% of cortex, and d) extensive damage involving more than 50% of the cortex.

The autoradiographs were scanned using the GS-800 Calibrated Densitometer (Quantity One, Bio-Rad). Results are expressed as n-fold increase over control as mean±SEM.

Differences between groups were assessed by one-way analysis of variance, followed by post-hoc Bonferroni or Dunnett test, or Mann-Whitney test, as appropriate. $P<0.05$ was considered significant. Spearmen test was done to correlate TIF and gremlin mRNA expression. Statistical analysis was conducted using the SPSS statistical software (version 11.0).

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IV. DISCUSIÓN

La investigación de los mecanismos moleculares implicados en la fibrosis y en la reparación del tejido renal buscan mejorar los tratamientos existentes para retardar la progresión de la ERC y el desarrollo de estrategias para prevenir su aparición. Los resultados presentados en esta tesis muestran los complejos mecanismos implicados en la regulación de la TEM inducida por AngII y el rol de nuevos mediadores como Gremlin en la fibrosis renal.

1. La Angiotensina II induce transición epitelio-mesenquimal en el riñón *in vivo* e *in vitro*

La infusión sistémica de AngII en ratas durante dos semanas causó daño tubular discreto e inducción de TEM, reflejado por un aumento del marcador mesenquimal α-SMA y disminución del marcador epitelial E-cadherina. Varios autores han observado que AngII participa en el proceso de TEM *in vivo* en diversos modelos experimentales de daño renal.[15,60] Existen evidencias de TEM en humanos, tanto en nefropatías progresivas diabéticas como no diabéticas, donde se ha descrito aumento de producción de AngII y activación de miofibroblastos a nivel tubular.[90,92] En nuestros estudios en células túbulo-epiteliales en cultivo hemos demostrado que AngII causa supresión de E-cadherina y expresión *de novo* de α-SMA y Vimentina, lo que produce una pérdida de la adhesión epitelial y un cambio en la morfología celular desde un patrón epitelial a uno del tipo fibroblasto, lo que sugiere una fase de transición en el fenómeno dinámico de TEM, como ha sido observado por otros autores.[21] Todos estos datos demuestran que la AngII induce TEM en el riñón.

2. La Angiotensina II activa de forma directa, independiente de TGF-β, la ruta Smad en el riñón

Con el objetivo de investigar los mecanismos implicados en la inducción de TEM por AngII en el riñón, en primer lugar evaluamos la vía de señalización Smad. Nuestros estudios *in vivo* utilizando un modelo de infusión de AngII en ratas mostraron activación de la vía Smad tan precozmente como a las 24 horas, manteniéndose elevada hasta 2 semanas de estudio. Las ratas tratadas con AngII presentaron incremento en los niveles proteicos de Smad2/3 fosforilado, primer paso de activación de esta vía. Mediante la técnica de southwestern *in situ*, hemos observado mayor número de complejos de Smad activos, principalmente a nivel tubular y en algunas

células glomerulares. En células túbulo-epiteliales humanas en cultivo AngII indujo una rápida activación de la vía Smad a los 15 minutos de incubación, demostrado por varias técnicas experimentales (aumento en la fosforilación de Smad2/3, por western blot, translocación nuclear de Smad2 fosforilado y de Smad4, por immunocitoquímica y microscopia confocal, y un incremento en la actividad de unión de Smad al ADN, por EMSA). Estos datos demuestran que AngII activa la vía Smad a nivel renal *in vivo* y en células túbulo-epiteliales humanas en cultivo ***(FIGURA 9)***.

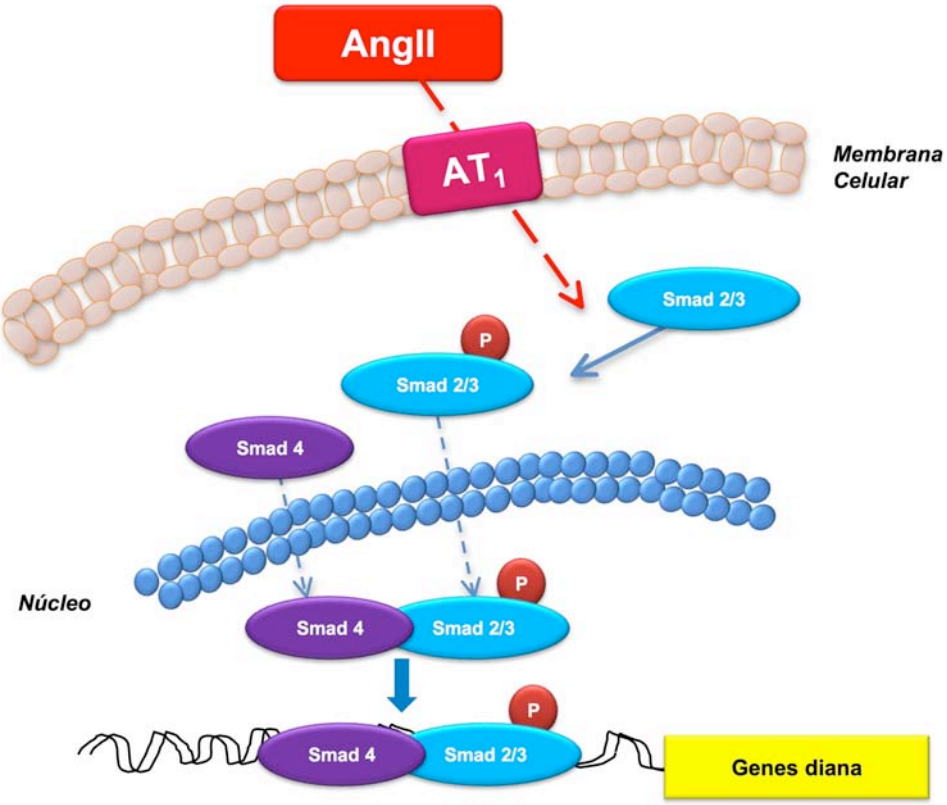

FIGURA 9. AngII activa en forma directa la ruta Smad en el riñón *in vivo* e *in vitro*.

Se ha descrito que AngII aumenta la expresión de TGF-β y su activación en células renales. Esta síntesis endógena de TGF-β está implicada en la producción de matriz extracelular.[169,94] En ratas infundidas con AngII durante 7 días se ha descrito un aumento de la síntesis de TGF-β a nivel renal.[61] Sin embargo, utilizando este modelo hemos observado que la activación precoz de Smad, inducida por AngII a las 24 horas de infusión, ocurre antes que el incremento de TGF-β observado a nivel génico a las 72 horas y a nivel proteico más tarde, a las 2 semanas. Estos datos sugieren que en riñón AngII causa una rápida activación de la vía Smad mediante un mecanismo independiente de TGF-β ***(FIGURA 10A)***. Para confirmar estos hallazgos se realizaron

estudios *in vitro.* Primero evaluamos el curso temporal de la activación de Smad y la inducción de TGF-β. En células túbulo-epiteliales en cultivo AngII aumenta la producción de TGF-β tras 24 horas de incubación, mientras que la activación de Smad ocurre después de sólo 15 minutos de estímulo. En segundo lugar, bloqueamos TGF-β utilizando un anticuerpo neutralizante frente a TGF-β activo y un inhibidor de la quinasa del receptor d e TGF-β. El bloqueo de TGF-β endógeno no afectó la translocación nuclear del Smad2 fosforilado y Smad4 ni la actividad de unión de Smad a ADN a los 15 minutos de incubación con AngII. Estos datos muestran que la AngII activa de forma directa e independiente de TGF-β, la vía de señalización Smad. Sin embargo, hemos observado que TGF-β media la activación tardía de la vía Smad, ya que el bloqueo de TGF-β disminuyó la transcripción dependiente de Smad inducida por AngII a las 24 horas ***(FIGURA 10B)***. Resultados similares han sido descritos en células vasculares.[125,162]

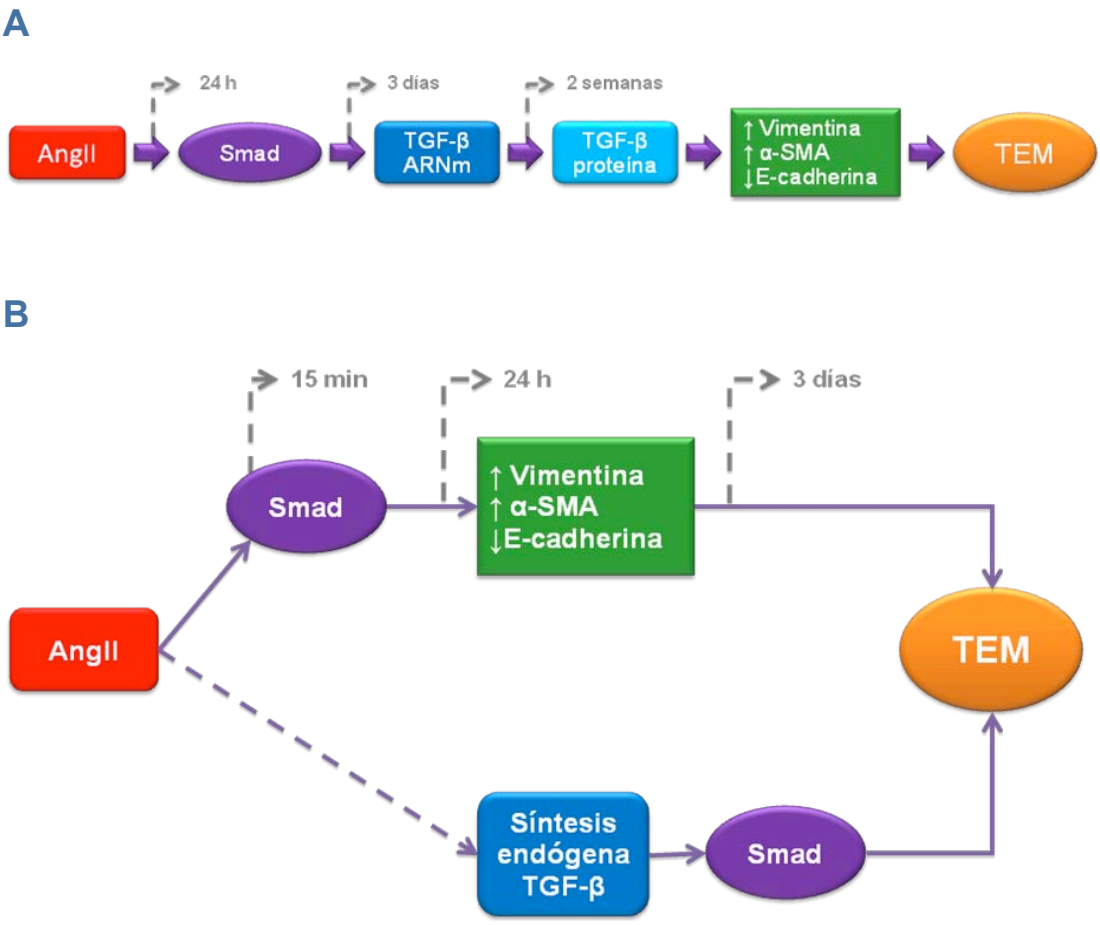

FIGURA 10. Mecanismos de acción de Smad y TGF-β implicados en la TEM causados por AngII *in vivo* ***(A)*** e *in vitro* ***(B).***

Existen diferentes tipos de proteínas Smad. Smad2 y Smad3 son mediadores específicos de las vías TGF-β/activinas, mientras que Smad1, Smad5 y Smad8 están relacionadas con la señalización de las BMPs.[85] Smad3 tiene un papel crítico en modelos de daño renal que inducen TEM, así como en metástasis de carcinomas invasivos.[98,123] Smad2 y Smad3 tienen papeles diferentes en la TEM inducida por TGF-β. La regulación de la expresión de CTGF y E-cadherina es dependiente de Smad3, mientras que MMP-2 es regulada por Smad2. Tanto Smad2 como Smad3 regulan α-SMA.[123] En CMLV, Smad3 está relacionada con la fibrosis vascular inducida por AngII.[162] Nuestros datos muestran que AngII activa las proteínas Smad2/3 en el riñón y en células túbulo-epiteliales en cultivo en forma similar a TGF-β.

Existe una interrelación entre MAPKs y Smad.[134] La sobreexpresión de los miembros constitutivamente activos de la cascada ras/MEK/ERK promueve las acciones de Smad3 en células mesangiales, en cambio en células epiteliales bloquea la acumulación nuclear de las Smads.[70] En CMLV, AngII activa la vía Smad mediante la activación de MAPKs.[125,162] En células HK2 encontramos que los inhibidores de las tres MAPKs (p38, ERK y JNK) disminuyeron significativamente la fosforilación de Smad2 inducida por AngII. Estos datos muestran una interacción entre las vías de señalización Smad y MAPK en células túbulo-epiteliales, sin embargo estudios futuros serán necesarios para evaluar si esto también ocurre *in vivo* a nivel renal.

3. La ruta TGF-β/Smad participa en TEM causada por AngII

TGF-β es el principal factor inductor de TEM a nivel renal.[33,67,152] En este estudio comparamos el efecto de AngII y TGF-β en la TEM en células túbulo-epiteliales humanas en cultivo. Observamos que a las 24 horas de incubación, AngII indujo expresión de marcadores mesenquimales y supresión de E-cadherina de forma similar a lo observado con TGF-β. Pasados 3 días se produjeron cambios morfológicos hacia un fenotipo mesenquimal, hecho que fue más marcado a los 5 días, no presentado diferencias significativas entre los efectos producidos por AngII y TGF-β, lo que sugiere que ambos factores tienen un efecto similar sobre TEM.

A continuación investigamos si TGF-β podría actuar como mediador de la TEM inducida por AngII. En células túbulo-epiteliales humanas en cultivo, el bloqueo de TGF-β endógeno no disminuyó la inducción de Vimentina a las 18 o 24 horas de incubación con AngII, demostrando que la inducción precoz de TEM mediada por

AngII es independiente de TGF-β. Sin embargo, el bloqueo de TGF-β disminuyó la inducción de Vimentina y la conversión fenotípica observada a los 3 días de incubación con AngII, lo que sugiere que la producción de TGF-β endógeno participa en el proceso de TEM a largo plazo ***(FIGURA 10B)***. La infusión sistémica de AngII provocó cambios en los marcadores de TEM tras 2 semanas, a este tiempo también se encontró activación renal de Smad y aumento de producción de TGF-β, lo que indica que en la infusión crónica de AngII, la activación de la vía Smad por TGF-β endógeno puede contribuir a la progresión del daño renal ***(FIGURA 10A)***.

La activación de la vía Smad es el principal mecanismo mediante el cual TGF-β induce TEM. En nuestros estudios *in vitro* demostramos que la vía Smad participa en la TEM inducida por AngII, para ello realizamos transfecciones transitorias con un vector que sobre-expresa Smad7, proteína que interfiere con la activación de Smad2 y Smad3.[125] El bloqueo de la activación de Smad previno la inducción de marcadores mesenquimales y los cambios morfológicos inducidos por AngII. En diversos modelos experimentales, incluyendo el de obstrucción ureteral unilateral y el de hipertensión experimental, la sobre-expresión de Smad7 atenúa la fibrosis renal.[49,66] Resultados similares se han encontrado en modelos animales de fibrosis peritoneal inducida por diálisis peritoneal en donde la sobre-expresión de Smad7 previene la fibrosis.[41,109] Sin embargo, este tipo de terapias no pueden ser utilizadas en humanos.

En la actualidad el bloqueo del SRAA, con inhibidores de la ECA o bloqueantes del receptor AT_1 son utilizados frecuentemente en patología renal humana con un efecto beneficioso probado en la protección de órganos diana, siendo las herramientas más efectivas para retardar la fibrosis.[169] Estos fármacos, además de disminuir las cifras tensionales, tienen un efecto antifibrótico adicional en comparación con otros antihipertensivos como beta bloqueantes o calcio antagonistas.[121,9] En células túbulo-epiteliales en cultivo observamos que la utilización de Valsartan, bloqueante del receptor AT_1, disminuyó la expresión de Vimentina y los cambios morfológicos inducidos por AngII. El uso de bloqueantes de AngII, además de inhibir las acciones de AngII, interfiere con la vía de señalización Smad/TGF-β, proporcionando una herramienta importante para dificultar las acciones de TGF-β y prevenir la pérdida funcional de tejido renal.

4. La activación de la cascada MAPK regulan la TEM inducida por AngII

Nuestro siguiente objetivo ha sido investigar el papel de la activación de proteínas quinasas en la TEM causada por AngII. Numerosos estudios han demostrado que la ruta de las MAPKs está involucrada en TEM y en fibrosis. AngII activa la cascada de señalización de MAPKs y a través de esta ruta regula múltiples respuesta celulares.[137,138] En células túbulo-epiteliales humanas en cultivo hemos observado que la inhibición farmacológica y específica de cada una de las tres MAPKs (p38, JNK y ERK1/2) previene de la conversión fenotípica de estas células epiteliales a miofibroblastos. En concreto, se inhibe la pérdida de E-cadherina observada después de tres días de tratamiento con AngII, y se disminuye, de forma significativa, la inducción de marcadores de TEM como Vimentina y α-SMA ***(FIGURA 11)***. La ruta de las MAPKs está implicada en la TEM, fibrosis y migración celular causada por TGF-β.[4,30,99] En diferentes tipos celulares las tres MAPKs, p38, ERK y JNK, participan en la TEM inducida por TGF-β, incluidas células túbulo-epiteliales de la línea celular NRK52E,[122] lo que demuestra un mecanismo intracelular común de respuesta para TGF-β y AngII.

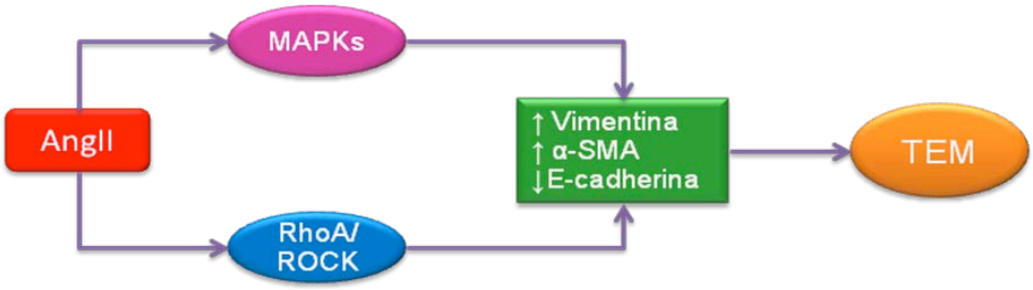

FIGURA 11. Las vías de señalización MAPKs y RhoA/ROCK regulan la TEM causada por AngII *in vitro*.

Estudios realizados en biopsias de pacientes con diferentes patologías renales sugieren que la activación de las MAPKs en células residentes e infiltrantes podría estar implicada en la progresión del daño renal. La activación de ERK1/2 se ha asociado con la proliferación celular y la disfunción renal,[84] mientras que en glomerulonefritis humanas, se ha observado una activación de p38 en células renales e infiltrantes, correlacionada con disfunción renal, proteinuria, infiltrado inflamatorio y lesiones proliferativas.[149] En modelos experimentales de daño renal se ha observado activación de JNK en podocitos, células endoteliales, macrófagos, células T y fibroblastos.[34] En algunos de estos modelos los inhibidores de MAPKs han

demostrado un efecto beneficioso sobre la evolución de las lesiones renales. El tratamiento con inhibidores de JNK redujo la acumulación de colágeno y la apoptosis en modelos de obstrucción ureteral e isquemia reperfusión.[34,150,163] Datos similares se han encontrado en riñones obstruidos de ratones deficientes en JNK1 y JNK2.[80] El bloqueo farmacológico de ERK1/2 previene la proliferación celular en glomerulonefritis mesangioproliferativa experimental.[11] El efecto de los inhibidores de p38 ha sido extensamente estudiado. En ratas hipertensas inhibidores específicos de p38 disminuyeron la proteinuria, la esclerosis y la migración de macrófagos intersticiales, vía supresión de la NAD(P)H oxidasa y mejorando la biodisponibilidad de NO, mostrando, aparte de la acción antihipertensiva, un efecto órgano-protector.[105,158] En ratas homocigóticas transgénicas con elevada síntesis de renina, la inhibición de p38 redujo la fibrosis, tanto glomerular como túbulo-intersticial, y la inducción de la expresión de α-SMA.[24,25] En ratas dobles transgénicas para renina y angiotensinógeno, la inhibición de p38 disminuyó la expresión renal de CTGF, TNF-α, IL-6, la infiltración de macrófagos y la fibrosis.[115] Recientemente, se ha desarrollado una nueva estrategia que inhibe p38 en las células del túbulo proximal, usando un conjugado del inhibidor de p38 SB202190 y una lisozima como transportador que se une de forma específica a las células túbulo-epiteliales. En el modelo de isquemia-reperfusión en ratas este compuesto redujo la fosforilación de p38 intrarrenal y la expresión proteica de α-SMA.[119] Estos datos sugieren que la inhibición farmacológica de la ruta de las MAPKs puede ser un importante avance terapéutico en patología renal.

5. La ruta RhoA/ROCK regulan la TEM inducida por AngII

Rho A participa en muchas respuestas de AngII, incluida vasoconstricción, cambios en el citoesqueleto e hipertrofia celular.[71,136] Numerosos resultados sugieren que la ruta de RhoA/ROCK está implicada en la etiología de la fibrosis renal. En nuestros experimentos *in vitro*, la transfección transitoria de un vector con un dominante negativo de RhoA o el uso de dos inhibidores de ROCK (Y-27632 y Fasudil) han demostrado claramente que la ruta de RhoA/ROCK regula la TEM mediada por AngII ***(FIGURA 11)***. La proteína G pequeña RhoA participa en la TEM mediada por TGF-β.[116,125] Los mecanismos de este proceso implican la degradación de RhoA por reclutamiento de la ubiquitina ligasa Smurf.[114] En modelos experimentales de daño renal, como el de obstrucción unilateral de uréter, la nefrectomía de ratas

espontáneamente hipertensas, el tratamiento con L-NAME o la infusión de AngII; la inhibición de ROCK mejoró el daño glomerular y túbulo-intersticial, y la fibrosis. En algunos de estos modelos la inhibición de ROCK disminuyó la sobreexpresión de los genes de α-SMA, TGF-β, CTGF y de proteínas de matriz.[63,149,140] Estas investigaciones apoyarían la idea de que los tratamientos que inhiben la ruta RhoA/ROCK puede ser una elección apropiada como estrategia terapéutica en enfermedades renales crónicas.

6. El CTGF es un mediador de la TEM inducida por AngII

A pesar de los numerosos esfuerzos que se han realizado para encontrar un biomarcador de la progresión en enfermedades renales crónicas, hasta ahora no hay un buen candidato. El CTGF está sobre-expresado en muchas enfermedades renales humanas y participa en la fibrosis y TEM inducidas por TGF-β.[177,118] CTGF actúa como mediador de la fibrosis renal inducida por AngII, regulando la producción de proteínas de matriz extracelular.[139,143] En el modelo experimental de daño renal por AngII, hemos descrito previamente que el CTGF se induce en el riñón a los 3 días de infusión de AngII y se mantiene elevado hasta las 2 semanas coincidiendo con el inicio de la TEM.[142] Estos datos se han confirmado en otros modelos experimentales, como nefropatia diabética, nefrectomía 5/6 en ratas, donde el aumento de CTGF se asocia con el aumento de la fibrosis, presencia de marcadores de TEM y la gravedad del daño renal.[142] En esta tesis, hemos observado que el bloqueo de CTGF con oligonucleotidos antisentido de CTGF o un inhibidor del receptor, disminuyó la inducción de Vimentina causada por AngII a 24 y 72 horas. Esto muestra que CTGF también contribuye a la TEM causada por AngII, actuando en las etapas tempranas y tardías del proceso. El papel de CTGF como mediador en la TEM inducida por AngII ha sido confirmado por otros autores.[21] Con estos datos podemos concluir que CTGF actúa como mediador de la TEM causada por factores profibróticos, como AngII.

En diferentes tipos celulares las proteínas quinasas MAPKs y ROCK están implicadas en la regulación de CTGF. En fibroblastos, la inhibición de ERK1/2 y JNK pero no la de p38 disminuyeron la sobre-expresión de CTGF causada por AngII,[76] mientras que en células mesangiales, se observó que únicamente el inhibidor de p38 SB203580 redujo el CTGF.[143] En fibroblastos renales en cultivo la activación de RhoA está implicada en la sobreexpresión de CTGF causada por TGF-β y AngII.[44,56] La

participación Rho en la regulación de CTGF ha sido descrita en muchos tipos celulares.[54,134] En este trabajo hemos observado que en células túbulo-epiteliales humanas AngII regula la activación de CTGF mediante la activación de las tres MAPKs (p38, ERK y JNK) y ROCK, mostrando una respuesta similar en la regulación de la TEM.

En pacientes con nefropatía diabética, se ha descrito que niveles elevados de CTGF en plasma podrían considerarse como un marcador temprano de la progresión de la disfunción renal en el riñón diabético,[157] y predicen la evolución de la enfermedad renal.[58] Estudios en pacientes con nefropatia IgA han observado que niveles elevados de CTGF y TGF-β en orina se correlacionan con el grado de daño tubulointersticial.[111] Por otro lado, en pacientes con daño cardíaco crónico, los niveles de CTGF en plasma dan información sobre la aparición de fibrosis miocárdica, pudiendo considerarlo como nuevo marcador de disfunción cardíaca.[64] Estos datos sugieren que CTGF podría ser un marcador de la fibrosis y progresión del daño en diferentes enfermedades.

El tratamiento con antagonistas del receptor AT_1 e inhibidores de la ECA disminuyen la expresión renal de CTGF y la fibrosis en varios modelos experimentales de daño renal.[136,139,165] Entre las nuevas opciones terapéuticas, el bloqueo del CTGF es una de las más prometedoras. Actualmente, los estudios de inhibición de CTGF están dirigidos hacia el desarrollo de oligonucleótidos antisentido, ARN de interferencia o anticuerpos neutralizantes que bloqueen CTGF endógeno. Estudios experimentales han demostrado que el bloqueo de CTGF, mediante oligonucleótidos antisentido, reduce la acumulación de MEC en ratones transgénicos para TGF-β1 sometidos a nefrectomía[113] y en ratones con nefropatía diabética.[73] En un modelo de fibrosis hepática, el tratamiento con un ARN de interferencia para CTGF vía vena intraportal atenuó la fibrosis hepática.[40] Todos estos datos sugieren que el bloqueo de CTGF endógeno podría ser una buena alternativa en el tratamiento de patologías renales asociadas a fibrosis.

En resumen, estos resultados muestran que AngII activa la vía de señalización Smad por un mecanismo independiente de TGF-β *in vivo* e *in vitro.* Además encontramos que las proteínas Smad participan en la TEM inducida por AngII. Este nuevo hallazgo sugiere que la activación de Smad puede participar en los efectos profibróticos mediados por AngII en las enfermedades renales. El CTGF destaca frente a TGF-β como mediador de la fibrosis y TEM causada por AngII, presentado además ventajas terapéuticas. Nuestros datos muestran que los inhibidores de

MAPKs y ROCK suprimieron la sobreexpresión de CTGF y TEM causada por AngII, sugiriendo que el bloqueo de estas rutas podría ser una importante elección terapéutica para enfermedades renales. Futuras investigaciones *in vivo* de los efectos de los inhibidores de quinasas en enfermedades renales crónicas podrían mejorar los actuales tratamientos para estos pacientes ***(FIGURA 12).***

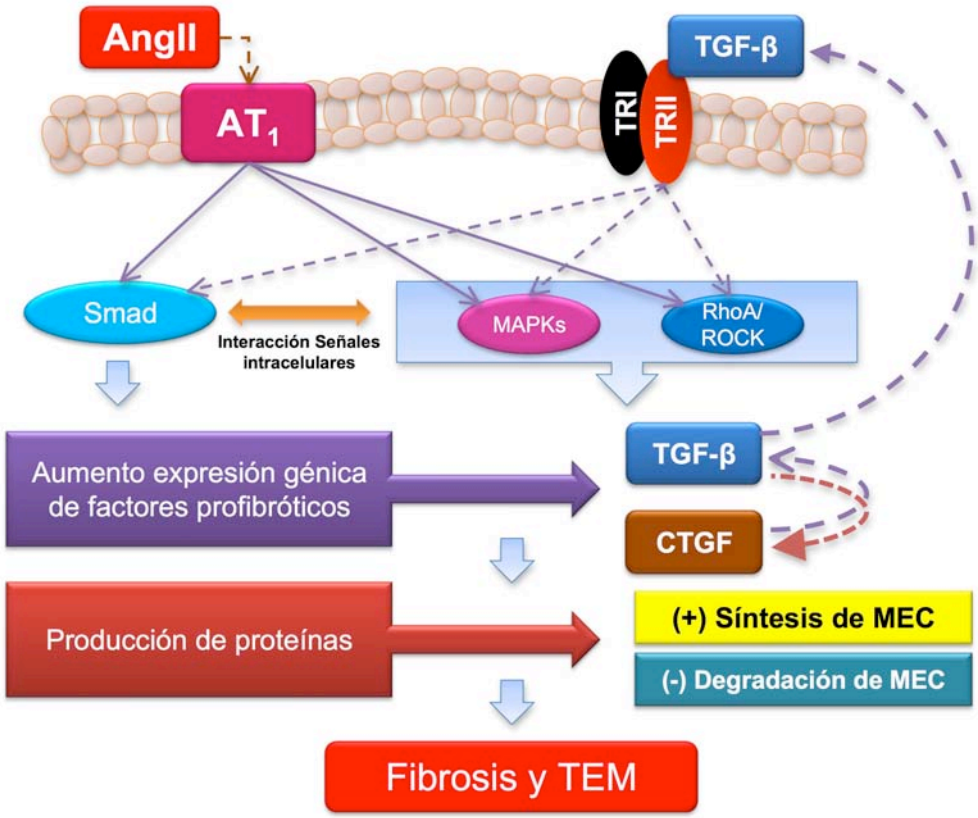

FIGURA 12. Mecanismos implicados en la TEM causada por AngII.

7. Gremlin es un nuevo mediador de la transición epitelio-mesenquimal y fibrosis renal

Nuestro siguiente objetivo fue evaluar el rol de Gremlin, antagonista de BMP-7, en el proceso de TEM y fibrosis renal. Estudios previos sugieren que Gremlin podría ser un mediador clave en la patogénesis de la nefropatía diabética. [27,68,87,100,126] En muestras de biopsias de pacientes con distintas nefropatías evaluamos la expresión génica y proteica de Gremlin, y su posible correlación con TEM y fibrosis renal. En primer lugar se estudiaron 30 biopsias renales de pacientes con glomerulonefritis pauci-inmune. Observamos marcada expresión de Gremlin, ARNm y proteína, en las células epiteliales parietales y monocitos presentes en las crescientes celulares y fibrocelulares de las biopsias estudiadas. También se observó importante expresión de Gremlin en células túbulo-epiteliales e intersticiales infiltrantes, lo que se correlacionó con el grado de fibrosis túbulo-intersticial. Posteriormente evaluamos la expresión renal de Gremlin en 16 biopsias de pacientes con nefropatía crónica del injerto, en las cuales detectamos sobre-expresión de TGF-β, en co-localización Gremlin ARNm y proteína, principalmente en áreas de fibrosis túbulo-intersticial. En los mismos túbulos encontramos disminución en la expresión de E-cadherina e inducción en la expresión de Vimentina y α-SMA. En un último estudio evaluamos la expresión de Gremlin en 125 biopsias de pacientes con glomerulopatías progresivas y no progresivas, encontrando sobre-expresión de Gremlin ARNm en todas las glomerulopatías progresivas estudiadas. Por el contrario, en la glomerulopatía no progresiva (enfermedad por cambios mínimos) no se observó inducción de expresión de Gremlin, tal como está descrito para el tejido renal normal. El gen de Gremlin se induce en las células renales residentes y en las células inflamatorias infiltrantes, principalmente en las áreas de fibrosis túbulo-intersticial. La expresión génica de Gremlin se correlacionó con el grado de fibrosis túbulo- intersticial. Estos hallazgos confirman el rol de Gremlin en nefropatía diabética y sugieren un rol patogénico global en las glomerulopatías progresivas humanas.

En cortes seriados de biopsias de pacientes con glomerulopatías progresivas y con nefropatía crónica del injerto encontramos evidencias de TEM en correlación con la expresión de Gremlin en células túbulo-epiteliales. Otros estudios clínicos utilizando biopsias renales humanas sugieren que la TEM tendría un rol importante en la patogénesis de la ERC. Se ha encontrado expresión de marcadores mesenquimales como Vimentina y Fsp1 en varias nefropatías progresivas, incluyendo nefropatía diabética, nefritis lúpica y nefropatía por IgA. [6,43,110,147] Más aún la expresión de estas

proteínas en las células tubulares epiteliales a menudo se correlaciona con un deterioro de la función renal.[78]

La presencia de Gremlin asociada a marcadores de TEM observada en las biopsias humanas no demuestra que las células epiteliales efectivamente sufrieron una transformación fenotípica y migraron al intersticio, ni que Gremlin sea el mediador del proceso, es por esto que realizamos estudios *in vitro*. La estimulación con Gremlin recombinante o la transfección con un vector de expresión de Gremlin en células túbulo-epiteliales humanas en cultivo indujo cambios morfológicos hacia un fenotipo de fibroblasto, con disminución en la expresión de E-cadherina y citoqueratina y sobre-expresión de Vimentina y α-SMA. Además, Gremlin indujo aumento en la expresión de fibronectina y MMP-9. Estos hallazgos se correlacionan con los cuatro eventos fundamentales en el proceso de TEM.[57,62,151]

Muchos estudios de diferentes grupos han demostrado que diversos factores crecimiento y citoquinas son capaces de inducción TEM *in vitro*.[176] Sin embargo, la obtención de una evidencia concluyente de que este proceso también ocurre *in vivo* ha sido mucho más difícil. El grupo de Neilson y colaboradores, empleando en un modelo de obstrucción unilateral del uréter, y células túbulo-epiteliales proximales modificadas genéticamente, demostró que un 36% de todos los fibroblastos FSP-1 positivos presentes en el intersticio se originaban de células túbulo-epiteliales proximales mediante el proceso de TEM.[57] Estudios recientes han mostrado que un número considerable de fibroblastos intersticiales provienen de células del endotelio capilar mediante un proceso que se ha denominado transición endotelio-mesenquimal. [74,174]

En la actualidad existe un intenso debate acerca de la contribución real que puede tener el proceso de TEM en la fibrosis renal en humanos, llegando a cuestionarse incluso su existencia en el riñón adulto. La pérdida de los marcadores epiteliales como E-caherina, ZO-1 y citoqueratina es un evento específico y fácil de evaluar. Sin embargo, el cambio fenotípico hacia fibroblasto se determina utilizando los marcadores mesenquimales disponibles en la actualidad, como Vimentina, α-SMA, Fsp1, que no son específicos de fibroblastos, ya que se expresan también en células inflamatorias y endoteliales. Se postula que las células epiteliales y endoteliales sometidas a un daño podrían sufrir un proceso de TEM incompleto, conocido como pre-TEM o TEM *in situ*, en el cual las células sólo expresarían uno o dos marcadores mesenquimales, pero no migrarían de su microambiente local.[43] Probablemente este

debate se mantenga, ya que por razones éticas los estudios en humanos utilizando técnicas genéticas están limitados.

Como hemos mencionado anteriormente TGF-β es conocido como el principal inductor de TEM durante la embriogénesis, cáncer y fibrosis.[96] En biopsias humanas de nefropatías progresivas y nefropatía crónica del injerto encontramos co-expresión de Gremlin y TGF-β en correlación con marcadores de TEM y fibrosis. Estudios previos han demostrado que TGF-β induce la expresión de Gremlin en células inflamatorias y renales residentes, lo se ha confirmado en esta tesis. [18,27,91] En células túbulo-epiteliales humanas en cultivo Gremlin indujo la expresión del ARNm y proteína de TGF-β. Además, hemos demostrado que TGF-β actúa como mediador de la TEM inducida por Gremlin. Estos hallazgos revelan la compleja interrelación entre Gremlin y TGF-β en el riñón, demostrando una retroalimentación positiva entre ambas proteínas para promover fibrosis y TEM.

Gremlin es antagonista de BMP-2, -4 y -7. Las BMPs pertenecen a la superfamilia de TGF-β y tienen un papel importante durante la morfogénesis promoviendo la diferenciación celular a través de la activación de proteínas Smad 1, 5 y 8; sus acciones están reguladas por proteínas antagonistas como Gremlin, el cual se une a las BMPs impidiendo su unión al receptor.[95,159] Estudios recientes utilizando agonistas e inhibidores de las BMPs han demostrado que estas proteínas favorecen la reparación completa del tejido dañado.[81,171] BMP-7 ha demostrado un efecto protector del tejido renal en varios modelos experimentales, inhibe la diferenciación celular y la apoptosis, además es capaz de preservar la integridad de la arquitectura glomerular e inhibir la acumulación de MEC a nivel mesangial.[75,79] Por lo tanto, podríamos hipotetizar que Gremlin ejerce sus acciones patogénicas por dos mecanismos: 1) mediante su interacción con la vía TGF-β/Smad, como hemos descrito en esta tesis, y 2) actuando como antagonista de BMP-7, lo que facilitaría las acciones de TGF-β, aunque esto no se ha demostrado en el riñón ***(FIGURA 13).***

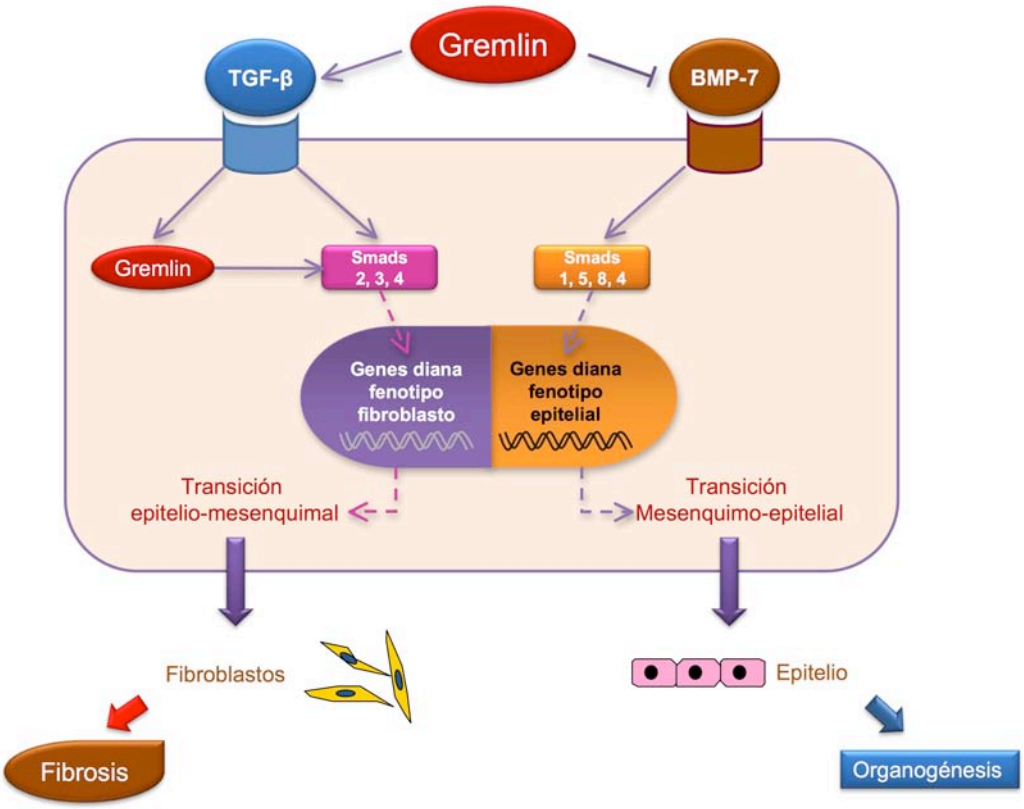

FIGURA 13. Mecanismos implicados en la TEM causada por Gremlin.

Estudios recientes han demostrado que Gremlin promueve la migración, proliferación y apoptosis de CMLV. Estas acciones estarían mediadas por factores de crecimiento como TGF-β, PDGF y AngII.[82,83] No existen estudios que evalúen la relación entre Gremlin y AngII a nivel renal. En ratas infundidas con AngII durante dos semanas observamos un aumento en la expresión génica y proteica de Gremlin. En células túbulo-epiteliales humanas en cultivo la estimulación con AngII indujo síntesis de Gremlin en forma similar a lo observado con TGF-β. Estos hallazgos sugieren que AngII es capaz de modular la síntesis de Gremlin a nivel renal, lo que potenciaría su rol como citoquina profibrogénica.

Existen pocos estudios que hayan investigado las acciones de Gremlin independientes de BMPs. Aunque no se ha descrito un receptor en la membrana celular se sospecha que Gremlin tendría acciones intracelulares directas. En líneas celulares tumorales Gremlin es capaz de suprimir la tumorigénesis mediante la sobre-expresión de p21 (Cip 1) y p42/44 MAPK.[19] En células endoteliales Gremlin induce la fosforilación de proteínas tirosinas quinasas, incluyendo la paxilina, la quinasa de adhesión focal y la MAPK ERK 1/2, estimulando la migración e invasión celular lo que

tendría un posible rol en la angiogénesis.[148] Además se ha demostrado que Gremlin inhibe la migración de monocitos mediante la unión a proteínas Slit.[20]

En el riñón la ruta Smad regula la TEM inducida por factores claves relacionados con la fibrosis renal tales como TGF-β [33,123,169] y, como se ha demostrado en esta tesis, AngII. En células HK-2 la estimulación con Gremlin recombinante indujo una rápida activación de la ruta Smad, caracterizada por la fosforilación de Smad2/3 y la traslocación nuclear del trímero Smad 2/3 fosforilado y Smad4 observado a los 20 minutos. La activación de la ruta Smad se confirmó en células transfectadas con el vector de expresión de Gremlin. La co-transfección de Smad7 y Gremlin en células túbulo-epiteliales inhibió la activación de la ruta Smad y la TEM. En esta tesis demostramos por primera vez la participación de la ruta Smad en las acciones intracelulares mediadas por Gremlin en células renales, en concreto en la TEM ***(FIGURA 13).*** La estimulación con Gremlin recombinante de astrocitos derivados del nervio óptico y células de la lámina cribosa en cultivo indujo la producción de MEC a través de la activación del receptor de TGF-β y la posterior fosforilación de Smad3, lo que sugiere un rol de Gremlin en glaucoma.[178] Se ha encontrado sobre-expresión de Gremlin ARNm en pulmones de ratones expuestos a asbesto, lo que se correlaciona con hallazgos de biopsias de pacientes con fibrosis pulmonar idiopática. Según estudios realizados en cultivos de células epiteliales bronquiales humanas, la sobre-expresión de Gremlin inducida por asbesto puede ser prevenida mediante el bloqueo del receptor de TGF-β y por inhibidores de la ruta MAPK/ERK.[101] Todos estos hallazgos sugieren que Gremlin podría ser un importante mediador de fibrosis en diferentes patologías.

En resumen, nuestros resultados sugieren que Gremlin es un factor patogénico común a diversas glomerulopatías progresivas humanas, y que el proceso de TEM inducido por Gremlin está regulado por la vía TGF-β/Smad.

V. CONCLUSIONES

A partir de los resultados obtenidos en la presente Tesis Doctoral, llegamos a las siguientes conclusiones.

1. La Angiotensina II induce transición epitelio-mesenquimal (TEM) en el riñón a través de la activación directa, independiente de TGF-β, de la vía Smad. La síntesis endógena de TGF-β está implicada en las respuestas crónicas de AngII en el riñón. Estos resultados, explican que los efectos órgano-protectores de los fármacos que inhiben AngII, van más allá de sus efectos hemodinámicos, y son debidos al bloqueo del eje TGF-β/Smad en el riñón.

2. La regulación de la transición epitelio-mesenquimal inducida por AngII es un proceso complejo e implica la interrelación entre diversos sistemas de señalización. La activación de proteínas quinasas, como la cascada MAPKs, regulan la activación de la ruta AngII/Smad y las respuestas celulares consecuencia de esta activación, contribuyendo así a la fibrosis renal. La activación de proteínas G pequeñas, como la vía RhoA/ROCK, también participa en la regulación de la transición epitelio-mesenquimal inducida por AngII.

3. El bloqueo de la producción endógena de CTGF inhibió la transición epitelio-mesenquimal inducida por AngII a tiempos cortos y largos, indicando que CTGF actúa como mediador de los efectos fibróticos de AngII en el riñón y apoyando la idea de ser una buena alternativa terapéutica para las enfermedades renales crónicas.

4. Gremlin se sobre-expresa en todas las glomerulopatías progresivas estudiadas, correlacionándose con el grado de fibrosis túbulo-intersticial, lo que sugiere que Gremlin es un factor patogénico común en enfermedades renales crónicas.

5. La síntesis renal de Gremlin está regulada por factores claves en la progresión del daño renal, como AngII y TGF-β.

6. En células túbulo-epiteliales humanas en cultivo, Gremlin indujo transición epitelio-mesenquimal a través de la activación de la ruta TGF-β/Smad.

En conjunto, estos datos muestran los complejos mecanismos implicados en los efectos profibrogénicos de la Angiotensina II y resaltan el papel importante que podría jugar Gremlin en la progresión de la enfermedad renal crónica, además de su rol potencial como nueva diana terapéutica para esta enfermedad que se ha convertido en un grave problema de salud pública a nivel mundial.

VI. BIBLIOGRAFIA

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ANEXOS

Los resultados presentados en esta tesis han sido publicados en:

Artículos originales:

- **Carvajal G,** Rodríguez-Vita J, Rodrigues-Díez R, Sánchez-López E, Rupérez M, Cartier C, Esteban V, Ortiz A, Egido J, Mezzano SA, Ruiz-Ortega M. Angiotensin II activates the Smad pathway during epithelial mesenchymal transdifferentiation. *Kidney Int.*;74(5):585-595, 2008

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Revisiones:

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- Rupérez M, Rodrigues-Díez R, Blanco-Colio LM, Sánchez-López E, Rodríguez-Vita J, Esteban V, **Carvajal G,** Plaza JJ, Egido J, Ruiz-Ortega M. HMG-CoA reductase inhibitors decrease angiotensin II-induced vascular fibrosis: role of RhoA/ROCK and MAPK pathways. *Hypertension*. 50(2):377-83. 2007.

- Sánchez-López E, Rodriguez-Vita J, Cartier C, Rupérez M, Esteban V, **Carvajal G**, Rodrígues-Díez R, Plaza JJ, Egido J, Ruiz-Ortega M. Inhibitory effect of interleukin-1beta on angiotensin II-induced connective tissue growth factor and type IV collagen production in cultured mesangial cells. *Am J Physiol Renal Physiol*. 294(1):F149-60. 2008.

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Los resultados de esta tesis han sido parcialmente presentados en las siguientes reuniones científicas:

- **Carvajal G,** Ruperez M, Sanchez-Lopez E, Rodriguez-Vita J, Rodrigues-Diez R, Esteban V, Mezzano S, Egido J, Ruiz-Ortega M. Angiotensin II causes epithelial mesenchymal transdifferentiation via activation of smad and mapk signaling pathways. Congreso ERA-EDTA. Glasgow. 2006. Comunicación Oral.

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- **Carvajal-González G**, Rodrigues-Diez R, Díez RR, Sánchez-Rodriguez J, Ortiz A, Egido J, , Mezzano S, Ruiz-Ortega M. Gremlin: nuevo mediador de la fibrosis y la transición epitelio mesenquimal renal. Congreso Nacional de la Sociedad Española de Nefrología. Donostia-San Sebastián. España. 2008. Póster.

- **Carvajal-González G**, Rodriguez-Vita J, Rodrigues-Diez R, Sánchez-López E, Díez RR, Sánchez-Rodriguez J, Mezzano S, Ruiz-Ortega M. La Angiotensina II activa la ruta de las Smad en riñon de forma directa e independiente de TGF-beta. Congreso Nacional de la Sociedad Española de Nefrología. Donostia-San Sebastián. 2008. Comunicación Oral.

- **Carvajal G**, Droguett MA, Burgos ME, Lavoz C, Ardiles L, Carpio D, Rodriguez-Diez R, Egido J, Ruiz-Ortega M, Mezzano S. “Gremlin: nuevo mediador de la fibrosis y la transición epitelio-mesenquimal renal”. Congreso Conjunto Nefrología, Hipertensión y Trasplante. Pucón, Chile. 2008. Comunicación Oral.

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Premios:

Congress EDTA. Young investigator Award a Gisselle Carvajal. Glasgow 2006.
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